THE BIOLOGICAL STANDARDISATION OF THE VITAMINS

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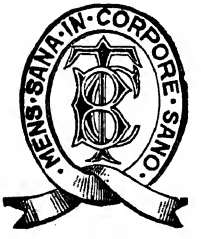

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PREFACE

THIS book has been written in the hope that it may be of service to those workers who are engaged in the determination of the vitamin potency of foods, of special preparations for therapeutic purposes, or of products obtained in the course of investigations on the chemical nature of the vitamins. In giving practical details of experiments, I have drawn largely from my own experience, but I have been fully aware that in many laboratories the technique must be quite as good as my own, and more suitable to the local conditions than my own could be. I have, therefore, dwelt largely on the principles which should underlie biological determinations of the vitamins, giving details of my own or others' technique only as I myself know them to be good. In particular I have stressed the need for International Standards of Reference and shown how they must be used so that a determination made in one laboratory may be reasonably concordant with one made in another laboratory, or in the same laboratory at a different time.

The book is divided into two parts. Part I deals with the practical side of the subject and may be used without reference to Part II if desired. Part II deals with the more mathematical treatment of the subject, the estimation of the accuracy of the results of experiments. It is a very elementary introduction to the study of statistical methods applied to vitamin determinations. It was written in the hope that even students who are not mathematically inclined will nevertheless read it and decide that the statistical treatment of results is really very simple after all. For students who wish to explore the possibilities further, there are available two excellent books, *The Methods of Statistics*, by Tippett (Williams and Norgate), and *Statistical Methods for Research Workers*, by Fisher (Oliver and Boyd).

No method of estimating vitamin E has been included since there is no international standard of reference for the factor yet.

I gladly take this opportunity of acknowledging my indebtedness to many people for training in various parts of my work, and of expressing my sincere thanks to them. To Professor J. C. Drummond I am indebted for six years of most generous teaching and guidance, not only in vitamin technique, but in the methods and aims of research generally; to Professor H. Steenbock for a very happy year of vigorous training during the tenure of a Rockefeller Travelling Fellowship at the University of Wisconsin; to Professor J. H. Burn for initiation into the principles of biological assay, and to Professor J. H. Gaddum and Dr. J. O. Irwin for many helpful discussions on statistical methods.

Acknowledgment is made to the Cambridge University Press and to the authors of the papers concerned for permission to reproduce Figs. 8, 9, 15, 17–21, 22, 26, 27, 35, 40, 42, 43 from the *Biochemical Journal*; to the Medical Research Council and His Majesty's Stationery Office for permission to reproduce Figs. 23, 31 from their Special Report Series No. 158; and to the Health Organisation of the League of Nations for permission to reprint the whole of Appendix I of this book from the *Quarterly Bulletin of the Health Organisation.*

KATHARINE H. COWARD.

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BIOLOGICAL STANDARDISATION OF THE VITAMINS

PART I

CHAPTER I

THE GENERAL PRINCIPLES WHICH GOVERN THE BIOLOGICAL METHODS OF DETERMINATION OF THE VITAMINS

EVERY worker on the biological activity of the vitamins is familiar with the difference in response given by different animals to the same dose of any particular preparation of a vitamin, and it is evident that the greater the number of animals used for testing a dose of vitamin, the more nearly will the average response obtained approach the true average response to that dose. A much more serious difficulty than this has, however, to be faced. Not only do individual animals vary in their response to a given dose but the response of the whole stock of animals to a given dose has been shown by several workers to fluctuate gradually over long periods of time. This means that the potency of a preparation can never be measured simply by measuring the response of animals to a certain dose of it at any time, for six months later, or a year or five years later, the response of animals to the same dose would almost certainly be greater or less than the original response. Moreover, it is obviously impossible to expect that the response of animals to a given dose in one laboratory would be equal to the response of animals to the same dose in another laboratory. To measure the potency of a substance simply by measuring the animal response would lead to widely different determinations and the greatest possible confusion.

The solution of this difficulty is easily available. For each of the vitamins A, B_1, C and D a standard of reference has been adopted for international use. A particular preparation of each vitamin has been made and presented to the Health Organisation of the League of Nations. The biological activity of a certain weight of each preparation is accepted as a unit of activity. The standards are held by the National Institute for Medical Research, London, acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations. Any worker desiring to use the standards may obtain them on application to the distributing agent in his own country. It is, however, of the utmost importance that each worker should understand how the standards must be used. The purpose of a standard of reference is to provide a measure, in terms of the unit adopted, of the biological activity of a substance. A dose of a substance which produced a response equal to that produced by, say, three units of the Standard would be said to contain three units of activity and the activity would be expressed in units per gramme. But since the response to a given dose of a vitamin fluctuates, it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the Standard *at the same time.* In every test half the animals available should be given a dose (or doses) of the test substance and the other half should be given a dose (or doses) of the International Standard. Only in this way, comparison with the Standard by simultaneous testing, can the activity of a preparation be determined and stated in terms of the International unit. Certain workers are credited with having obtained a sample of a standard, tested a dose (or doses) of it on their own stock of animals, noted the response and never used it again; but any dose of material which at any subsequent time produced a response equal to that produced originally by a dose of Standard was declared to have a potency equal to that dose of Standard. These workers have completely ignored the fluctuations of their colony; indeed they have probably never recognised them. Hence the whole value of the use of the Standard has been lost and erroneous determinations of vitamin potency have been made. It cannot therefore be stated too strongly that whenever a vitamin determination is required, simultaneous tests of

the Standard and test substance must be made, and since the response of animals to a dose of Standard varies as much as the response to a dose of vitamin in any other form, equal numbers of animals must be used for the Standard and for the test substance.

Descriptions of the International Standards of reference for vitamins A, B_1, C and D are given in Appendix I, pp. 213–222. Further details of the methods of using them are given in the appropriate chapters.

When standards of reference for other vitamins become available, the same principles will govern their use as govern the use of the standards already adopted, for it is impossible to imagine an animal reaction that does not show fluctuations from time to time throughout the whole colony, or one that does not differ in different laboratories in which conditions of lighting, heating and diet must necessarily vary very much.

CHAPTER II

ANIMALS SUITABLE FOR THE DETERMINATION OF VITAMINS BY BIOLOGICAL METHODS

1. Rats.
   A. The housing of the rats.
   B. The diet of the rat colony.
   C. The strain of rat suitable for vitamin tests.
2. Guinea-pigs and Pigeons.
3. References.

RATS are used for the determination of vitamins A, B_1 and D. Pigeons and sometimes rice birds are used for the determination of vitamin B_1, and guinea-pigs are used for the determination of vitamin C. All workers on vitamins are agreed that it is essential to control the breeding and feeding of rats which are to be used for vitamin tests and that this can best be done in a room given up to this purpose in the building where the tests are to be carried out. It is not necessary to control the breeding and feeding of pigeons before using them for tests, nor is this necessary for guinea-pigs provided that the animals are obtained from a clean and healthy stock.

1. Rats

A. The housing of the rats.

Daylight is not necessary for the breeding of rats ; thus a room without windows can be usefully employed in housing the rat colony. If the room available for the rats has windows which do not face north, they should be covered with blinds. This is to prevent irradiation of the rats at irregular intervals with ultra-violet rays which might make their reserves of vitamin D vary too much. If the room faces north, the use of blinds is probably unnecessary.

The whole of the interior of the room should be washable, and the floor should slope down to a drain so that it may be easily cleaned. There should be as little woodwork in the room as possible ; one bench or table may be allowed, but it should

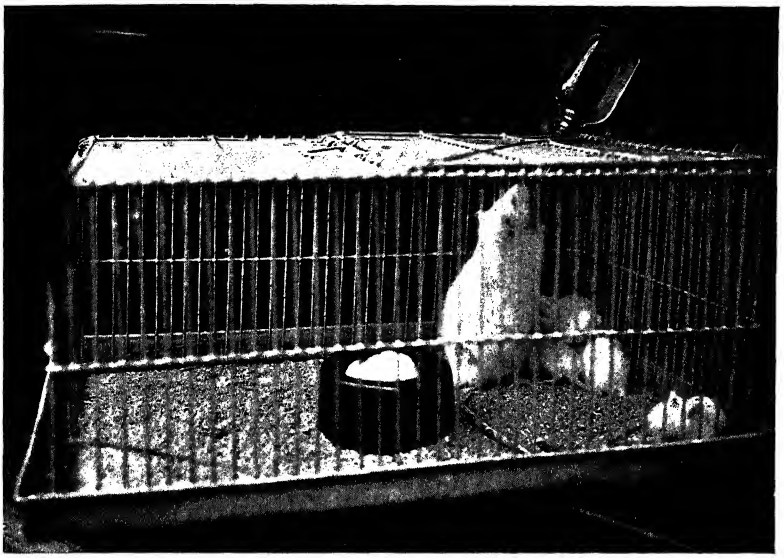

FIG. 1.—A useful type of rat cage.

FIG. 2.—Simple fitting for a rat room.

[*To face page* 4.

be made of teak with metal supports. A good supply of hot and cold water is necessary and a large sink and draining board are useful, even if all cage-cleaning is done in another room.

The room should be kept at a fairly constant temperature of about 65–70° F., though this is not so necessary for the rat colony as it is for the experimental rats, many of which must be considered sick animals. Ventilation is necessary both for the rats and for the workers. Draughts, of course, are undesirable. Adequate artificial lighting is essential.

It is necessary to keep the rats in cages which are large enough to allow them a fair amount of movement, but the cages must be not too heavy to be moved easily. One simple form consists of a galvanised iron tray, 3″ deep with a loose upper part (sides and top only) made of wire, in some form of open-work, about 8″ high, standing in the tray. The sides of the tray provide protection from draughts and prevent the scattering of the bedding, while the open-work top allows access of fresh air. The top should have a hinged door, either in the top to open upwards or in the front to open forwards. It should be large enough to allow the worker to get a hand and arm inside the cage for catching a rat or changing a food-pot. The " overall " size for such a cage is about 12″ (long) × 18″ (deep) × 8″ (high) (Fig. 1).

The stands for these cages can easily be made of hollow gas-tubing, jointed to any measurement desired. There should be enough space between the tiers to allow the cages to be pulled in and out easily and also to allow a water-bottle (described below) to rest on top. Thus about 12″ should be allowed between tiers. Stands may be arranged against the walls of the animal room, and, if space allows, back to back down the middle (Fig. 2). Enough space should be left somewhere in the room to allow for changing of cages ; that is the transferring of the rats from a dirty cage to a clean cage which is most easily done on a fairly low table.

There are many types of water-bottles in use in different laboratories ; some are simple and some are elaborate. The essential point is that the rat should have clean water every day and that it should be supplied in an inverted vessel so that it cannot be fouled. A simple form is an ordinary glass bottle with a neck large enough to allow the inside to be easily cleaned occasionally and provided with a straight delivery tube

wide enough at one end to fit into the neck of the bottle with a piece of rubber tubing, and drawn out at the other end to a diameter small enough to hold in the water when the bottle is inverted, and yet large enough to let the rat get the water easily by licking it. The tube of the water-bottle is fixed between the wires of the upper part of the cage.

Bedding should be supplied liberally. It may consist of sawdust or wood-shavings, preferably of pine-wood or deal. It has been stated that shavings of teak are harmful to rats.

When several rats are kept in one cage of the size mentioned above, the cages should be changed at least twice a week. Where a doe has a cage to herself awaiting parturition, and during the lactation of her young, it is enough to change her cage once a week. Dirty cages should be removed from the rat room, scraped, washed with soapy water and rinsed well in clean water. They may then be stacked to dry overnight. In a healthy colony sterilisation of the cages is not necessary. If occasional rats are suspected of being unhealthy, their cages should be washed in 5% lysol and well rinsed in clean water, for lysol is toxic to rats as well as to bacteria.

Food-pots should have inturned upper edges to prevent the rat from scratching out the food on to the floor of the cage. They should be washed thoroughly at least once a week.

Many elaborate arrangements have been designed for the housing and feeding of experimental animals, but in most places it is possible to find suitable structures and utensils made locally which are very much cheaper than many which are obtainable elsewhere. The cost of running a rat colony is necessarily high but sound economy can be practised by using material which is easily available.

B. **The diet of the rat colony.**

It must not be assumed that a diet found to be satisfactory for the growth and reproduction of rats in one laboratory will necessarily be equally satisfactory in another laboratory. The diet must be good enough for growth and reproduction but must be low enough in vitamin content to ensure low reserves of these factors in the young. To strike a balance between these two needs is sometimes very difficult for it is not possible yet to bring up a colony on a purely synthetic diet. All diets contain some substances such as yellow maize, wheat, dried

milk or fresh milk and even casein which must vary in composition. The sample of any one of these obtainable in one part of the country may be different from the sample obtainable in another and the difference may be just great enough to make a diet known to be satisfactory in the one part, unsatisfactory in the other. Moreover, it is to be expected that a diet which has proved satisfactory even for a year or two in one place may need some modification from time to time. It is very unlikely that the average reserves of any factor in a stock of rats will remain stationary over a long period of time, even with a diet kept as nearly uniform as possible. A diet which the writer (Coward, Cambden and Lee, 1932) has found satisfactory for some nine years consists of:

Yellow maize, ground finely	65·0 parts
Whole wheat, ground finely	20·0 ,,
Caseinogen, light white (B.D.H.)	9·0 ,,
Dried brewer's yeast	5·0 ,,
Sodium chloride	0·5 ,,
Calcium carbonate	0·5 ,,
Dried milk	20·0 ,,
Iron citrate *	0·1 ,,
Potassium iodide *	0·1 ,,

In addition to this mixture which is supplied to the rats *ad lib.*, some green food (generally watercress) is given in amount about 4g. per rat per week and also fresh uncooked liver or beef, about 4g. per rat per week. An extra 5% of dried yeast is added to the diet for the lactating does.

It is an interesting but unexplained fact that although the diet has supported a vigorous colony of albino rats for some ten generations, yet three different attempts to establish a colony of black and white rats on this diet have failed, even though the young piebald rats had been obtained from three different sources.

A diet devised by Steenbock (1923) and found satisfactory through many generations of rats in his Wisconsin laboratories has the following composition:

Yellow corn, ground finely	76·0%
Linseed oil meal	16·0%
Crude casein	5·0%
Ground alfalfa	2·0%
Sodium chloride	0·5%
Calcium carbonate	0·5%

* Additions made since the publication of the paper.

In addition to this, the rats received a generous supply of fresh cow's milk, brought straight to the laboratory from the University farm and given to the rats within 2 hours of milking. Certain cows belonging to the herd of the University were always fed on a winter ration to keep the vitamin D potency of the milk low. The diet for the young does was supplemented with 5% butter.

However, when the writer adopted this diet for her colony at its beginning in 1927, the rats in the second generation failed to grow normally and though they produced some litters, they did not wean them. Even when the diet was supplemented with extra vitamins, A, B complex, D or E or A+D, no improvement was found and it was concluded that the diet, as made in the London laboratory, was lacking in some unknown factor which was supplied in the diet as made in the Wisconsin laboratory. It seemed very probable that it was the milk (the ordinary London pasteurised supply) which was inferior to the fresh milk of the Wisconsin supply. This has never been proved by experiment though later work in this laboratory and in others lends weight to the probability. Kon (1931) who had worked in the Wisconsin laboratories and knew the value of the Steenbock stock ration, found that the ingredients available in his laboratory at the State School of Hygiene, Warsaw, did not make an adequate diet; nor was he able to get good results with diets compounded according to the formula of Professor Evans of California University.

Again, Smith and Anderson (1929) reported failure of a diet based on the formula that Sherman and Campbell (1924) had found adequate for many generations of rats, their only modification of the Sherman diet being the addition of calcium carbonate (1% of the wheat constituent), of fresh lettuce *ad lib.* and, for the lactating does, 9g. of tested dried yeast per week. It is impossible to believe that any of these additions lowered the value of the ration and yet it did not produce such good results as the Sherman diet in the Columbia laboratories.

All of these failures are open to the suggestion that it was not the diet which was at fault but some other item in the management of the colony. Coward again put her form of the Steenbock diet to the test, however, when she had her own stock diet giving good results, so that the only difference in treatment of the two lots of rats was in their respective diets

Again the Steenbock diet, as made with materials available in London, failed.

Thus there is abundant evidence that a diet which has been found completely satisfactory in one laboratory may not be found satisfactory in another when made with materials that are pardonably thought to be the same but which in reality may be very different. Each worker who has a colony of rats must work out for himself a suitable diet from the materials available in his laboratory. Until this is done, it is advisable to make careful records of the breeding of the individual rats, and compare them with records of other workers. Otherwise, it is only too easy to think that a colony is behaving badly or well when actually it may be the reverse.

C. **The strain of rat suitable for vitamin tests.**

The domesticated form of the rat, *Mus norvegicus*, is suitable for experimental purposes. In particular the albino rat, *Mus norvegicus albinus*, is bred by many investigators, though others breed the piebald variety.

The albino rat has been bred for experimental work for a great many years at the Wistar Institute, Philadelphia. Greenman and Duhring (1931) have written a full account of the procedure there.

Inbreeding of carefully selected rats is advantageous for the production of a uniform strain. Brother-sister mating in a healthy stock seems to have no unfavourable results (King, 1918), but it is often inconvenient to keep strictly to this method and it is certainly unnecessary for vitamin studies.

The rats may be mated when the does are about 150g. weight, and the bucks somewhat heavier. Does can produce and lactate their young when mated at a lower weight than 150g. but their milk supply may then be insufficient for the litter and the first mating may be wasted. The ingestation period is normally 21–22 days. Lactation should be carried on for 21–25 days though the young can sometimes be weaned at 14 days if needed. A doe can produce and rear 5–6 litters with an average of 5–6 rats per litter when fed on a diet such as is described above. It is often advisable to allow a doe to rest for two weeks between the weaning of a litter and the next mating. These figures were obtained from the records of Coward, Cambden and Lee (1932) for their colony of albino

rats fed on the diet described above which was somewhat restricted in quality to ensure low reserves of the vitamins in the young. Many rat breeders whose only aim has been to produce large numbers of healthy young rats have evidence of much more prolific breeding than this, but their diet is probably richer than any of those described in this chapter.

It is of great value to keep full records of the breeding of a colony, at least until one is satisfied with the diet which has been adopted for it. The rate of growth of the rat should be determined from birth to the first mating by weighing weekly, then averaging bucks and does separately, and drawing composite growth curves from the averages. It may be of interest for some purposes to note the age and weight of each doe when her vagina opens. It is certainly worth while to examine vaginal smears daily in order to ascertain whether the stock is having œstrous cycles regularly. This is done by inserting a small spatula into the vagina of the rat, pressing the edge against the vaginal wall and withdrawing a little of the contents. This is rubbed off on to a drop of water on a slide and examined under the low power of a miscroscope without staining. The œstrous cycle in the rat has been fully investigated by Long and Evans (1922). They find it to recur every 5 or 6 days in the normal animal and to show the following stages :

a. Dicestrus, numerous leucocytes and a variable proportion of nucleated cells for about 3 days.

b. The beginning of œstrus—no leucocytes, numerous nucleated cells—for about 6 hours.

c. Œstrus, the best time for mating—nucleated cells and keratinised epithelial cells for about 24 hours.

d. The ending of œstrus—numerous keratinised cells for about 24 hours (Fig. 3).

The cycles are often irregular in unhealthy rats. Dietary deficiencies may be detected by irregularities in the cycles before they are detected in any other way. A shortage of vitamin A in the diet leads to the presence of keratinised cells in the vagina daily, whereas a shortage of vitamin B (the complex) (Evans and Bishop, 1922) or of vitamin B_1 (unpublished results of the writer) leads to a complete absence of keratinised cells.

The best time for mating a doe is when the vaginal contents consist of a mixture of nucleated and keratinised cells. or of

keratinised cells only. The doe will not receive a buck at any other time. When copulation has taken place, spermatozoa are generally found in the vaginal contents the next morning. A plug may or may not be found loosely filling the lower end of the vagina. From a healthy buck, the spermatozoa are very numerous and often found in large sheafs. They may even be still motile when examined. From about the 12th to the 16th day after a successful copulation there is a certain amount of bleeding into the vagina, and the smear consists of a little clot of blood, quite easily recognised by the naked eye. If this is absent no litter will be born. There are no cycles during ingestation. Records of matings, the occurrence or absence of spermatozoa and plug in the vagina, the occurrence of blood clots in the vagina, and the ingestation period should all be recorded for each doe. Loss of weight after the occurrence of a blood clot in the vagina and subsequent failure to produce a litter indicates that resorption after implantation of fertilised ova has taken place. It may be due to a lack of vitamin E in the diet.

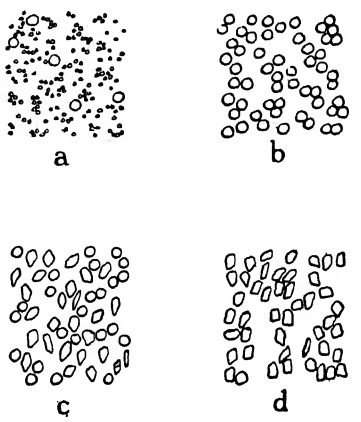

FIG. 3.—The contents of the vagina during the œstrous cycle.

Coward, Cambden and Lee (1932) recorded the ingestation period of 358 litters together with the success or failure of the does in lactating these litters, during the time when the Steenbock stock diet (as made of materials available in their laboratory) was slowly proving itself inadequate. The colony was obviously lacking in some dietary essential, and it was shown that the longer the ingestation period the smaller was the percentage of litters weaned (Fig. 4).

Thus by keeping full records of the growth and reproduction of the rats of a colony, some information may be obtained as to possible deficiencies before such deficiencies would otherwise become apparent. Even if no deficiencies are detected, it will have been well worth the time expended on making the

observations to be sure that the colony is behaving in a normal way. Variations must be expected. The behaviour of the rats of a healthy colony with regard to growth and reproduction are no more uniform than any other animal reaction, and a know-

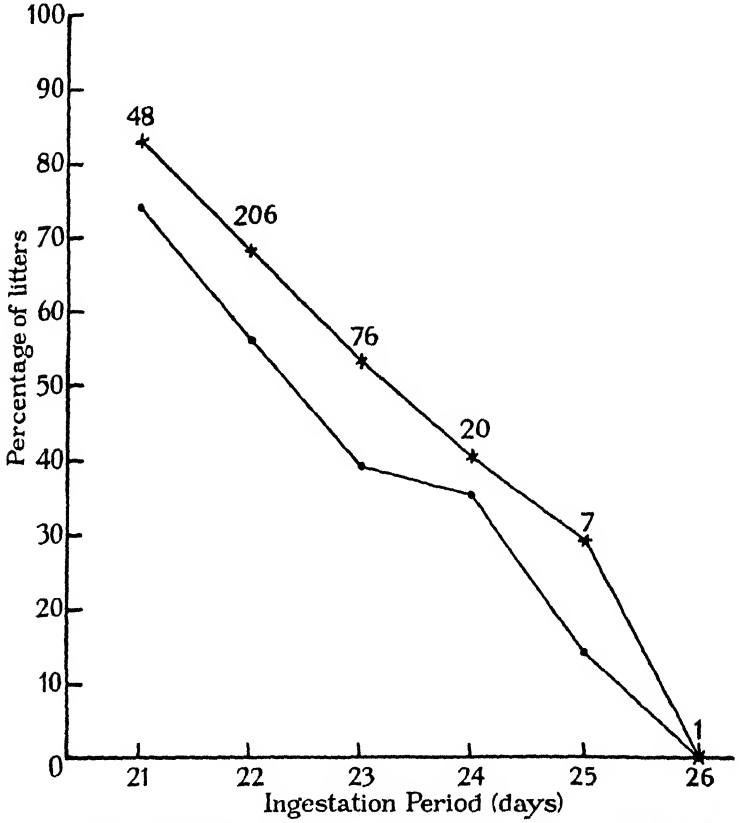

FIG. 4.—The relation between the mortality of young rats and the length of their ingestation period.

Number at each point indicates number of litters born.
* = percentage that lived one week.
● = percentage that were weaned.

ledge of their variation is exceedingly useful for comparison with the variation encountered in all vitamin experiments.

2. Guinea-pigs and Pigeons

Since vitamin C is made by the rat, that animal cannot be used for vitamin C determinations. Guinea-pigs are used for

this work, and since vitamin C is not stored to any appreciable extent in the guinea-pig's body, it is not necessary to limit the amount of this factor in the food of the stock of guinea-pigs for work on vitamin C. Guinea-pigs may be obtained from any reliable, healthy stock as needed, kept for two or three days in the laboratory to recover from possible disturbances of a journey and then used for experiment at once.

The same procedure may be adopted for pigeons which are to be used for experiments on vitamin B_1 and also for rats which are to be used for this purpose, since neither rats nor pigeons store appreciable amounts of this factor.

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CHAPTER III

THE DETERMINATION OF VITAMIN A

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5. References.

1. The International Standard of Reference and Unit of Vitamin A Activity

In 1931 the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations recommended that carotene be adopted as a provisional International Standard of reference for vitamin A. Seven workers in different laboratories each prepared 4–5g. of carotene which were sent to the National Institute for Medical Research, London, where they were pooled, recrystallised and distributed in ampoules of 10mg. each. This preparation served as a standard of reference until further knowledge of the chemistry of the lipochromes revealed the fact that this was not a pure substance but a mixture of substances. In 1934, therefore,

the Permanent Commission on Biological Standardisation recommended the substitution of pure β-carotene for the International Standard of reference and asked Professor Karrer to prepare the necessary sample. This was done and the preparation presented to the League of Nations. It was sent to the National Institute for Medical Research, London, where its distribution was undertaken by the Department of Biological Standards. The preparation actually sent out to workers is a solution of the sample of β-carotene in coconut oil of a concentration of 500 units per gram.

The unit of vitamin A activity is defined as that amount of activity contained in 0·6γ of the International Standard of β-carotene. This is the amount contained in 2mg. of the solution as issued by the National Institute for Medical Research. It is equal in biological activity to 1γ of the preparation of carotene provisionally adopted in 1931. The unit of the later preparation therefore is equal in biological value to the unit of the earlier preparation. The weight of β-carotene, 0·6γ, was chosen so that the biological value of the unit should not change. Hence a preparation which was found to contain, say, 1,000 International units of vitamin A activity when tested against the provisional Standard, should also be found to contain (within the limits of experimental error) 1,000 International units when tested against the new Standard.

A. **The dilution of the Standard of reference for dosing.**

Since a daily dose of two or three units of vitamin A is sufficient to bring about a response in rats as they are usually prepared for the test, the International Standard of reference is too concentrated to use as issued. The oil to be used for its dilution must be chosen with great care. Some oils slowly destroy β-carotene as shown by a loss of colour of the solution. Obviously such oils are unsuitable for dilution of the Standard. The International Standard is made up in coconut oil and, for its dilution, a sample of coconut oil should be used which has recently been shown to have no destructive effect on β-carotene dissolved in it. This may be determined by matching the depth of yellow colour of a weak solution (say, 0·003%) against the yellow (and red) glasses of a tintometer or against a 0·5% solution of potassium bichromate in a colorimeter, before and after storage at 37° C. for seven days; if the loss of colour is

not more than 10% the coconut oil may be considered satisfactory as a diluent of the Standard solution of carotene.

A summary of the evidence on which the recommendation was based by the authority issuing the International Standard was made by Hume and Chick in the Special Report No. 202 of the Medical Research Council. It was suggested in this report also that the greater activity of β-carotene dissolved in coconut oil than of β-carotene dissolved in some other oils (*e.g.* one sample of arachis oil, one sample of olive oil) might indicate a more complete utilisation by the rat of β-carotene from this solvent than from others, but Coward (1936), from somewhat indirect evidence, has shown that this is probably not the explanation of the apparently lower activity of β-carotene in some of the oils than in others.

The possibility of the vitamin A activity of cod liver oil being enhanced or diminished when the oil is diluted with other oils for dosing should also be recognised. An early result of the writer indicated a greater activity of vitamin A when the cod liver oil was diluted with coconut oil than when diluted with olive oil; but a recent comparison with different samples of coconut oil, olive oil and arachis oil has given very concordant results (unpublished results). With 19–20 rats in each of three groups, the rats of the different groups being given a daily dose of 1mg. of a particular sample of cod liver oil dissolved in coconut oil, olive oil and arachis oil respectively, the potency of the cod liver oil in the different solvents was found to be in the ratio 0·98 : 1·35 : 1·30, from which it may be concluded that these particular samples of coconut, olive and arachis oils had the same influence on the vitamin A of the cod liver oil.

B. Need for a simultaneous test on the Standard of reference whenever a vitamin A determination is made.

In making a biological test for any vitamin, it is of the greatest importance that a simultaneous test be made on the Standard of reference. Not only do individual animals vary in response to a given dose of any vitamin, but the whole of the rats of a colony may show gradual fluctuations in their response to the same dose of a vitamin, generally without a recognisable cause. This may be illustrated for vitamin A by the figures obtained by Coward, Key and Morgan (1933) in

successive tests of a sample of cod liver oil (named A in their laboratory) which was used as a standard of reference pending the adoption of an International Standard (Table I).

TABLE I

VARIATION IN THE AVERAGE RESPONSE OF GROUPS OF RATS (FROM 9 TO 16 RATS IN EACH GROUP) WHICH HAD CEASED TO GROW ON A DIET DEFICIENT IN VITAMIN A AND HAD THEN BEEN GIVEN A DAILY DOSE OF 2MG. COD LIVER OIL A FOR 5 WEEKS.

Test.	Date.	Average increase in weight of equal numbers of male and female rats in 5 weeks.*
1	June–July, 1931	10·3
2	August, 1931	11·9
3	October, 1931	14·9
4	November, 1931	7·3
5	January–February, 1932	19·0
6	April–May, 1932	14·0
7	June, 1932	27·7
8	July–August, 1932	34·0
9	August, 1932	22·8
10	August–September, 1932	13·4
11	September, 1932	23·6
12	September–October, 1932	18·6

It is evident that, however the differences in mean increase in weight of groups of rats may be interpreted in such experiments as this, the difference between 7·3g. and 34·0g. must be regarded as significant. If interpreted by means of the curve of response to be described later, which has been shown to be applicable to results such as these, the mean increase in weight of 34·0g. obtained in July–August, 1932, appears to show that the cod liver oil then had five times the vitamin A value that it had in the previous November. This is, of course, impossible. The result shows quite clearly that some influence was at work, presumably on the whole colony, which made the response to a given dose of vitamin A greater at one time than another. No reason for this was found. A similar, even greater, fluctuation

* In each group the average results from the male and female rats were determined separately; and then the two results from each group were averaged to find the average for the group supposing it to have consisted of equal numbers of male and female rats. This is the figure really required for comparisons, and it is better to obtain it in this way than to discard one or two rats to make the numbers of males and females equal in a group, for one would never know which rat or rats could fairly be discarded.

has been found since in the same laboratory with another cod liver oil (Z), and again no cause of the fluctuation could be found. The two results amply demonstrate the need for making a simultaneous test on a standard of reference whenever a substance is to be tested for vitamin A. Other results indicate a similar need of a standard of reference for each of the other vitamins.

One point in regard to the use of a standard of reference in all biological tests may be mentioned here. It is essential to use about the same number of animals in the test on the Standard as in the test on the substance whose vitamin potency is to be determined. That is, the same degree of accuracy is needed in determining the influence of the Standard at the time of the test as in determining the influence of the unknown substance. When "litter mate" comparisons are being made, it is obvious that each litter should be represented equally in each group of animals.

C. The general arrangement of the test for the determination of the vitamin A content of a substance in terms of the International Standard.

There are two ways of arranging a test for determining the vitamin A content of a substance in terms of the International Standard. The rats may be given a vitamin A-free diet until their reserves of vitamin A are exhausted and they show signs of vitamin A deficiency. They are then given daily or bi-weekly doses of the test substance or of the Standard of reference for a specified period of time. On the other hand they may be given the vitamin A-free diet plus doses of the test substance or Standard from the beginning of the experiment. In the first method, often spoken of as the curative or therapeutic method, the response to the vitamin is measured by the animals' behaviour from the day they were given the first dose of test substance or Standard. In the second method, often spoken of as the preventative or prophylactic method, the response to the vitamin is measured by the animals' behaviour from the beginning of the experiment. Each method has advantages over the other method which will be discussed later.

Whichever method is used, equal numbers of animals should be used for testing the Standard and the substance under examination. When increase in weight is the criterion adopted

for the comparison, the proportion of male rats to female rats should be the same in the two groups, though the number of male rats need not equal the number of female rats. If this arrangement cannot be made, an adjustment of the calculation of the result can rectify it.

The rats to be used for testing the Standard are divided into two groups as nearly alike as possible, and the rats for the substance under examination are divided similarly. The two groups for the Standard are then given doses of the Standard in the ratio 2 : 1 and those for the substance under examination are given doses in the ratio 2 : 1 also if some information of its possible potency has already been gained, but in the ratio 3 : 1 or 5 : 1 if no such information is available. The object of the experiment is to find a dose of the substance under test which gives a result nearly equal to the result given by one of the doses of the Standard. This arrangement of the test can be applied to both the curative and the prophylactic methods, but in the latter method a group of " no dose " rats should be included and the test carried on for a few weeks after the " no dose " rats have died.

If many substances are to be examined for vitamin potency an economy of time and labour may be effected by the preliminary construction of a curve of response relating the response to the amount of vitamin given. This, however, can only be done for tests of the " curative " type in which rats are reduced to states of vitamin depletion as nearly equal as possible before being used in a test. A large number of animals are divided into four or five similar groups of, say, 30 animals, in each group. The groups are given doses of some substance of uniform content (*e.g.* a particular sample of cod liver oil, the vitamin potency of which is not necessarily known) in the ratio 1 : 2 : 4 : 8 : 16, every rat of any one group being given the same dose. The results are averaged and plotted against the doses given. When this has been done, future tests can be carried out on two groups of rats only, one being given the Standard and the other a dose of test substance which is likely to be somewhere near the potency of the dose of Standard chosen. The ratio of the potencies of these doses is, then, not the ratio of the averages of the responses in the two groups, but the ratio of the abscissæ of the curve of response corresponding to these average results.

In order to average the response of different animals to the same dose, some method of measuring the response must be adopted. For the "increase in weight" method, the weights of the different animals are easily averaged. For the cure of ophthalmia the numbers of days taken to effect a cure are averaged, but the condition of the eyes that may be considered the starting point for the "curative period" and the condition when they may be considered cured must be clearly recognised by the worker concerned. It is not necessary that workers in different laboratories should adopt exactly similar conditions as their criteria. Comparison with the Standard of reference by a simultaneous test makes that unnecessary.

In the "increase in weight" method, the responses of the male and female rats in each group may be averaged separately and two curves of response constructed, one for male rats and one for female rats. It is useful to have these two curves for comparing results when it has not been possible to have equal numbers of male and female rats in each group.

A curve of response may be drawn freehand as smoothly as possible through, or nearly through, the points determined experimentally and plotted on graph paper. A more reliable way is to determine the shape mathematically if possible and then to draw the curve to fit the shape so determined. Many biological curves of response are logarithmic in shape, *i.e.* if the results are plotted against the logarithms of the doses they fall on a straight line. If a series of results obtained from different doses in any biological experiment shows a curvilinear relationship to the dose given, it is always worth while to plot those results against the logarithms of the doses given. If the points then lie on, or nearly on a straight line, the best straight line through these points can be obtained as in the following calculation:

Example.—The mean increases in weight in 3 weeks of five separate groups of male rats which had become steady in weight on a diet deficient in vitamin A and which had then been given doses of 0·25, 1·0, 1·5, 2·5 and 7·5mg. respectively of cod liver oil per rat per day, were —11·2, 7·3, 9·5, 16·8 and 33·4g. respectively. The curve of response relating the increase in weight in 3 weeks of rats so treated is found thus:

	Increase in weight in 3 weeks, y	Daily dose of cod liver oil, mg	Log. of daily dose of cod liver oil, x	Deviation from mean $\bar{x}$, $x-\bar{x}$	Product of y into $x-\bar{x}$, $y(x-\bar{x})$	Square of deviation from mean $(x-\bar{x})^2$
G. 1	−11·2	0·25	$\bar{1}$·3979	−0·7715	+8·6408	0·5952
2	7·3	1·0	0·0000	−0·1694	−1·2366	0·0287
3	9·5	1·5	0·1761	+0·0067	+0·0636	0·0000
4	16·8	2·5	0·3979	+0·2285	+3·8388	0·0522
5	33·4	7·5	0·8751	+0·7057	+23·5704	0·4980
	5)55·8		5)0·8470		34·8770	1·1741
	11·16 $=\bar{y}$		0·1694 $=\bar{x}$			

$$b \text{ (the slope of the curve)} = \frac{\Sigma y(x-\bar{x})}{\Sigma(x-\bar{x})^2} = \frac{34{\cdot}8770}{1{\cdot}1741} = 29{\cdot}71.$$

The straight line is represented by the equation

$$y=\bar{y}+b(x-\bar{x})$$
$$=11{\cdot}16+29{\cdot}71(x-0{\cdot}1694)$$
$$=6{\cdot}13+29{\cdot}71x \text{ (Fig. 5).}$$

Hence the curve relating increase in weight and dose (mg.) of C.L.O. given is represented by the equation

$$y=6{\cdot}13+29{\cdot}71 \log. x \text{ (Fig. 6).}$$

It is obvious that curves of response can be used for the interpretation of results only when these have been obtained from animals as nearly as possible in the same condition as those which were used for the construction of the curve. For example, a curve of response to dose of vitamin A, which had been obtained by plotting mean increases in weight in 3 weeks in rats which had ceased to grow on a diet deficient in vitamin A and had then been given doses of vitamin A, could not be used for interpreting results which had been obtained on rats whose reserves of this factor had been only partially depleted.

Moreover, a curve of response obtained in one laboratory is not necessarily applicable to animals in another laboratory even if these have been prepared, as far as one can tell, in exactly the same way as the animals of the curve were prepared. (See Chapter VII for discussion of different curves of response.)

2. Preparation of Rats for Vitamin A Determinations

Certain arrangements for vitamin A tests can be made whichever criterion of vitamin A depletion is to be used.

A. Animals suitable for the test.

It is essential to have rats for vitamin A tests whose reserves of vitamin A are small, otherwise they may grow to maturity on a vitamin A-free diet. In a curative test, they

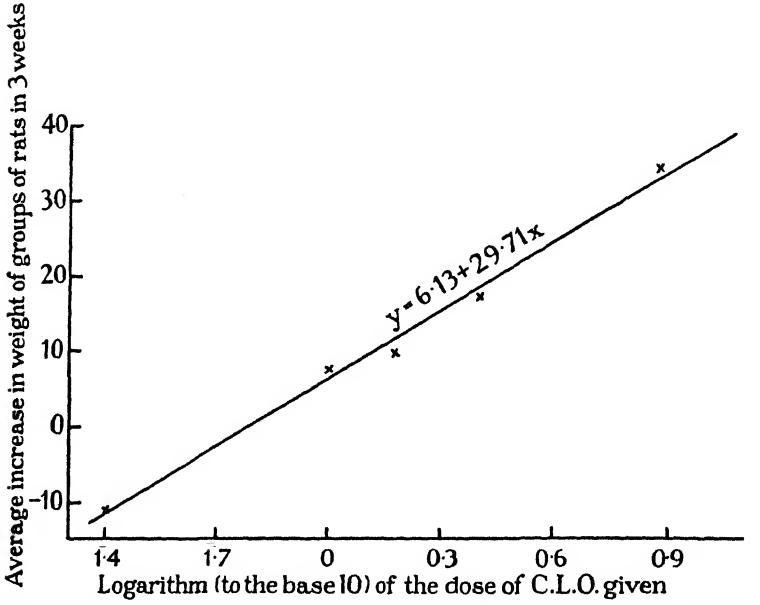

FIG. 5.—Curve of response relating increase in weight of male rats in 3 weeks and the logarithm (to the base 10) of the dose of cod liver oil given.

would not give a starting point for the test and in a prophylactic test they would not show any difference in effect between the vitamin A-free diet and that diet plus a dose of the unknown or of the Standard. The diet of the stock colony must therefore be regulated accordingly. Details of a suitable diet for a colony which is intended to supply rats for vitamin A tests have been given in Chapter II.

B. Housing of the animals.

The room in which experimental animals are kept should be reasonably large and airy. Overcrowding should be avoided,

partly for the sake of the rats and partly for the sake of the workers in the rat room. Several medium-sized rooms are preferable to one large one for various obvious reasons. The usual considerations of cleanliness apply to experimental animals as well as to the colony. The temperature of the room should be kept as nearly constant as possible at 20° C. A cold night may have disastrous results on the rats of a vitamin A experiment.

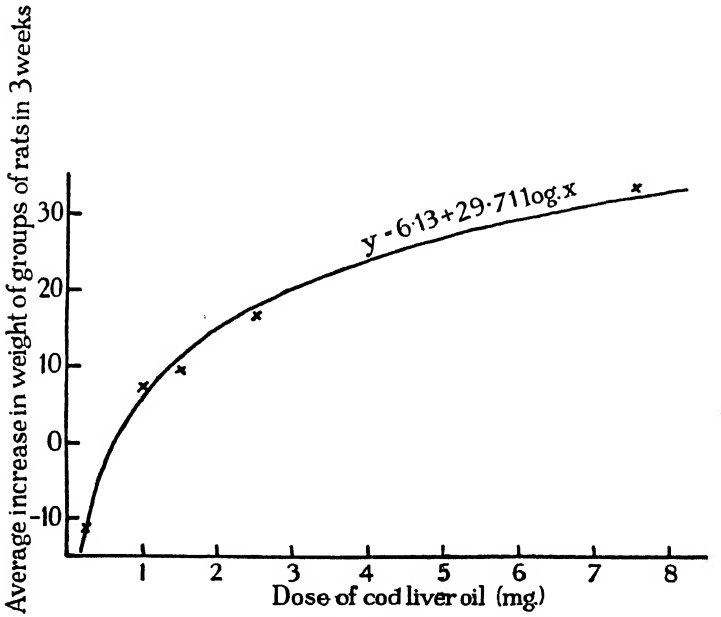

FIG. 6.—Curve of response relating increase in weight of male rats in 3 weeks and the dose (in mg.) of cod liver oil given daily.

The cages of animals which are to hold a whole litter of 8–10 rats during the depletion period should be about 12″ long, 18″ deep (back to front) and 8″ high, and made of galvanised iron. The base should be solid with a rim 1–2″ high standing up all round it. The top part should be quite separate from the base and made of wire.

During the test period when each rat is given a dose of test substance or of Standard, it should have a separate cage of dimensions at least 12″×9″×6″ (high).

Bedding may consist of shavings or sawdust. Wire screens

to raise the rats from the sawdust are not necessary in experiments on vitamin A, though some workers find it convenient to use wire mesh screens with sheets of blotting paper or newspaper in the tray. These are absorbent and easily renewed every day.

A north aspect is probably the best for a room in which vitamin A tests are to be carried out, but it is quite possible that direct sunlight would be without effect on rats fed on a diet deficient in vitamin A but well supplied with vitamin D.

C. **Vitamin A-free diet (basal diet).**

The vitamin A-free diet must be as nearly as possible free from vitamin A, and must not vary in this respect though the use of the Standard of reference will to a great extent control variations in results due to unavoidable and slight variations in the vitamin A content of the basal diet. Moreover, the diet must contain an abundance of all other factors known to be necessary for the well-being of the experimental animal. This point should be proved to each worker's complete satisfaction before any experiments are begun, and also at intervals later whenever any serious change is made in the stock of any ingredient of the diet. It may easily be done by giving the diet to, say, two or three litters of young rats, until their reserves of vitamin A are exhausted and then giving each one a large supplement of the purest form of vitamin A available. Growth should be resumed at a rate equal to that of the stock colony. If not, the diet should be investigated before any further experiments are undertaken. It was an experience of this kind that led Coward, Key and Morgan (1929) to the detection of what was probably a previously unrecognised vitamin.

A diet which has been found suitable for vitamin A tests in several laboratories consists of :

Caseinogen	15%
Dextrinised rice starch ..	73%
Dried brewer's yeast ..	8%
Salt mixture (Steenbock's 40)	4%

This is supplemented by giving each rat 8–10 International units of vitamin D per week. A solution of irradiated ergosterol of known strength is diluted so that one drop of the solution,

0·02g., contains 8–10 units of vitamin D. One drop of the solution can conveniently be given directly into each rat's mouth once a week.

The kind of caseinogen used in this diet is of great importance. Most samples of caseinogen probably contain a trace of vitamin A which is carried down with it from the milk from which it is prepared. Some workers remove most of the fat and vitamin A by extraction with alcohol and ether, but this is a laborious process and very costly; other workers heat the caseinogen in thin layers in an oven at 105° C. for 24 hours, stirring it occasionally to expose fresh surfaces to the air and thus to destroy, by oxidation, the traces of vitamin A.

Four samples of caseinogen have been found suitable for vitamin A tests by the writer:

1. "Light white" casein, sold by the British Drug Houses Ltd., but not prepared by them. Information as to the method of preparation has been refused by the makers.

2. A sample of caseinogen kindly prepared by the British Drug Houses Ltd., by the text-book method of precipitation by dilute hydrochloric acid from diluted skimmed milk.

3. A sample of caseinogen also kindly prepared by the British Drug Houses Ltd., by precipitation with alcohol.

4. Two samples of sodium caseinate, "Physiological caseinate," prepared for the market for vitamin tests by the Glaxo Co.

The casein, which for some reason still undetermined, was found to be unsuitable for vitamin tests was the kind previously sold by the Glaxo Co. as vitamin-free casein. It is certain that some workers found this form of casein satisfactory. It is equally certain that other workers besides the writer did not. In view of the fact that many animals were able to make full use of it as a protein, it does not seem to be probable that it was a biologically poor protein. The possibility is suggested that "light white" casein contained, as an impurity, some substance (which may be another vitamin) in which the Glaxo vitamin-free casein was lacking; and that the rats which grew well on the Glaxo casein did so because they held reserves of the substance on which they could draw, whereas the rats which did not grow well had no reserves. A full account of the work has already appeared in the literature and has received support from Mapson (1932, 1933) who has been able to make an extract

of liver which apparently makes good the deficiency of the Glaxo vitamin-free casein. It indicates that this may be a purer, not a poorer, form of casein than the others which have been found satisfactory. Whatever may prove to be the explanation, the practical outcome of the work is to emphasise the necessity of ensuring that the basal diet for a vitamin test should contain abundance of all factors necessary for the well-being of the animal except the one factor for which the test is being made. This can only be determined by supplementing the diet with the purest form available of the missing vitamin to see whether " normal " growth can be induced in rats whose reserves of that particular vitamin have been exhausted.

Almost any kind of starch is suitable for a vitamin A-free diet, but if the diet is used in the dry state, the starch, being very finely powdered, collects in the rats' fur, which is unsatisfactory. Moreover, raw corn starch which is freely used in vitamin work in America, is not easily digested by rats. Also, rats which have eaten uncooked starch, particularly potato starch, have been found to be subject to a condition known as refection in which undigested starch is excreted in the fæces. For all these reasons, it is desirable to cook the starch somewhat. Some workers mix the starch with enough water to make it stand in solid lumps and then autoclave it. Others mix the starch with enough water to make a thin paste, then boil it to burst the starch grains, then spread the paste on flat tins and bake it over hot pipes until brittle. A simple method found satisfactory by the writer is to mix the starch with enough water to make solid lumps, break these into small pieces and bake them in flat baking-tins in an ordinary gas cooking-stove until brittle. Whichever method of cooking is adopted the partially dextrinised starch must be ground to a fairly fine powder for mixing in the diet. It will keep in this state almost indefinitely.

The dried yeast should be tested for vitamin B (complex) potency, sample by sample, from any one source until it has been shown to be of reasonably constant potency. It must be ensured that the quantity incorporated in the diet allows for " normal " growth when abundance of vitamin A is also supplied.

Various salt mixtures have been used by different workers with equal success. Steenbock's mixture, No. 40 (Steenbock

and Nelson, 1923), reproduces as nearly as possible the salt content of cow's milk. It consists of:

Sodium chloride (NaCl)	23·36 parts
Magnesium sulphate ($MgSO_4.7H_2O$)	24·6
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	35·8
Dipotassium hydrogen phosphate (K_2HPO_4)	69·6
Calcium phosphate ($Ca_2H_2(PO_4)_2.4H_2O$)	68·8
Calcium lactate. $5H_2O$	15·4
Iron citrate. $6H_2O$	5·98
Potassium iodide (KI)	0·16

Osborne and Mendel's salt mixture (Osborne and Mendel, 1919) which many workers have used, consists of:

$CaCO_3$	134·8g.	Citric acid + H_2O ..	111·1g.
$MgCO_3$	24·2g.	Fe citrate. $1\frac{1}{2}H_2O$ ..	6·34g.
Na_2CO_3	34·2g.	KI	0·02g.
K_2CO_3	141·3g.	$MnSO_4$	0·079g.
H_3PO_4	103·2g.	NaF	0·248g.
HCl	53·4g.	$K_2Al_2(SO_4)_4$	0·0245g
H_2SO_4	9·2g.		

The chemicals used were analysed and allowance was made for moisture, etc. The acids were mixed and the carbonates and ferric citrate added to them. The traces of KI, $MnSO_4$ and $K_2Al_2(SO_4)_4$ were added as solutions of known concentrations. The final resulting mixture was evaporated to dryness in a current of air at 90–100° C. and ground to a fine powder.

One salt mixture (185) used by McCollum, Simmonds and Pitz (1916) which has also been used by other workers consists of:

NaCl	0·173g.	
$MgSO_4$ (anhydrous)	0·266g.	
$NaH_2PO_4.H_2O$ ..	0·347g.	
K_2HPO_4	0·954g.	
$CaH_4(PO_4)_2.H_2O$ ..	0·540g.	
Ca lactate	1·300g.	(Iron citrate 1·0g. was added by some workers.)

A comparison of these three shows how very varied may be the salt mixture given to experimental animals, all of these different mixtures being apparently equally good.

A generous supply of fresh water should be given. Tap water from the laboratory is generally suitable. There is no need to use distilled water.

3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin A

It is recognised by all workers on vitamins that vitamin A is stored in the animal body in varying amounts, and that when

the animal is fed on a diet deficient in vitamin A its reserves of that factor are gradually exhausted, and certain symptoms appear in the animal, some of which are apparently typical of vitamin A deficiency. If the animal continues to be deprived of vitamin A the condition becomes more severe, and in some 10–20 days from the first appearance of the symptoms the animal dies. Post-mortem examination reveals various lesions which were not recognisable in the living animal, *e.g.* a large proportion of rats which die from a deficiency of vitamin A are found to have abscesses at the base of the tongue, or they may have congestion of the lungs or various intestinal disorders. These conditions, however, are not obvious in the living animal and, indeed, are not specific for vitamin A deficiency. The symptoms which are useful in experiments on the determination of vitamins are those which can be recognised in the living animal before the condition has become too severe to be cured, unless the criterion to be measured is simply the survival period after the giving of a dose of vitamin A.

The most useful criteria for determinations of vitamin A in food substances are :

(*a*) Increase in weight of young animals.

(*b*) Xerophthalmia.

(*c*) Irregularities in the desquamosa of the vagina of the rat.

When young rats whose reserves of vitamin A are not very great are given a diet deficient in that factor, they continue to put on weight for some time but eventually cease ; they may develop a painful condition of the eyelids and later of the eyes (though curiously this does not happen in some laboratories) and, in female rats, after the vagina has opened, a smear of the contents of the vagina is found to contain keratinised epithelial cells continuously day after day, instead of at the normal intervals of about five days.

The difficulties of making any of these measurements are obvious. A rat does not suddenly cease to grow on a particular day nor is the onset of xerophthalmia a sudden occurrence ; several days must pass before it is certain that a rat's weight has become steady, or that the condition of the eyelids has reached the required degree of severity. There must be therefore some uncertainty as to the exact day to terminate a prophylactic experiment or to begin a curative one. A similar

difficulty is met again towards the end of the curative test. Even in measuring increase in weight the fluctuations in weight of an animal throughout any day prevent the measure from being as accurate as it might be expected to be. The vaginal smear method appears to offer a sharp " end-point," but it is not always a simple matter to decide whether the keratinised epithelial cells can be considered really to have disappeared from the vaginal contents, for a few stray ones sometimes occur during the dioestrous period of normal rats. Also since keratinised epithelial cells appear for 2 days during the normal œstrous cycle, if the normal occurrence is followed by, or preceded by, the abnormal occurrence of keratinised epithelial cells day after day, there might easily be an error of two days in the measurement. This particular difficulty could be overcome by ovariectomising the rats, when œstrous cycles would cease and the normal occurrence of keratinised epithelial cells in the vagina would cease also.

The difficulties of determining " end-points " by any of the criteria so far described appear to indicate that the death of the animal, the only certain end-point, must be the best criterion of all. No work on this method has been published, and it might produce good results, but probably the length of time required to wait for the death of all animals would make the method cumbersome and unpractical.

A. Increase in weight as the criterion for the determination of vitamin A.

α. The " Curative " Method

(*a*) *The preparatory period.*—Young rats weighing as little as 25–30g. may on weaning be given the vitamin A-deficient diet. If they do not grow at all, rather older rats may be used. The best rats for the test are those that become steady in weight on a vitamin A-deficient diet at about 80–90g. They should be weighed once a week for the first 3 weeks of the preparatory period, then twice a week until they begin to grow more slowly, then every day or two when they cease to put on weight. After this they decline in weight rapidly. Thus it is important to weigh them frequently in order to recognise the real steadying in weight. Until this happens all the rats of a litter may be kept in one cage, but as each rat becomes steady in weight it should be given a separate cage for the rest of the experiment.

The number of rats to be prepared is determined from the considerations outlined on pp. 19, 159. A few extra ones should be allowed as occasionally rats die through being left too long on the preparatory period.

Some workers insist on having isogenic pairs of rats, *i.e.* pairs of rats of the same sex from the same litter, one of each pair for the test on the Standard and one for the test on the substance under examination. In a prophylactic test this is, of course, essential and also in a test in which the reserves of vitamin A are only partly exhausted when dosing is first begun. But the writer has calculated (unpublished results) from large numbers of test animals in her laboratory that when the rats' reserves of vitamin A are exhausted and they become steady in weight, no greater accuracy is obtained from the use of isogenic pairs of animals than from rats of different litters. In her laboratory for several years a continuous supply of young rats has been prepared on a vitamin A-free diet and each day the rats which are considered to have become steady in weight are assigned in turn to the groups intended for the testing of (*a*) the Standard of reference, (*b*) the substance under examination, and often (*c*) another substance under examination. It appears to be of greater importance to start the test period of equal numbers of rats in the different groups on the same day than to wait a few days for "litter mates" to become steady for the test. Tourtellotte and Bacon (1935) have shown that temperature affects the response of rats to vitamin D; it is easily conceivable that it affects the response to vitamin A and that other conditions may affect it also. It is essential, then, that equal numbers of rats receiving the Standard and the test substance shall be subject to the same variations of environment, and the only way to ensure this is to assign equal numbers of rats to the different groups each day until the required number is obtained.

(*b*) *The test period.*—Dosing with the test substance and Standard respectively are continued for the same length of time, the rats being weighed once a week during this period. The length of the test period must depend on the desired accuracy of the result. The period used for a long time was 5 weeks, but the writer has shown that a result is only slightly less accurate if the test period is shortened to 4 or even 3 weeks (Coward, 1933). In 2 weeks only, the inaccuracy of the result

is much greater and no period less than 3 weeks can be recommended. In order to get the final weight at the end of the test period as accurately as possible, it is useful to weigh the rat on three successive days, the 20th, 21st and 22nd of the test and then take the average of the three weighings as the final result.

(*c*) *Dosing the rats.*—Until a few years ago doses of the Standard and of the test substance were given daily, but Coward and Key (1934) showed that the same result was obtained if the same total amount of Standard or of test substance was given in two doses per week only. The variation in response to this way of dosing was not greater than that in the daily dosing. (The values for σ for the male rats were 13·03 and 11·96 respectively, and for the female rats 9·45 and 9·81 respectively for the two ways of dosing.)

This method of dosing is convenient only when the substance tested is of such a high potency that an amount equal to 3 or 4 days' doses can be given on one day. Less potent preparations should be given daily. Substances of very low potency may be incorporated as a percentage of the basal diet, but if this is done records of food consumed daily must be made, *e.g.* 25g. of the substance to be tested may be finely powdered and mixed with 100g. of the basal diet, *i.e.* it is incorporated in the diet as 20% of it. A weighed amount of this (say, 10g.) is given to each rat, in a feeding pot that cannot be upset and that is large enough to prevent the rat from scratching out the food from it, each rat being housed in a separate cage. The amount not eaten should be weighed the next morning and made up to the previous weight by a fresh addition. Remnants not eaten should be thrown away only if moist or seriously soiled, for it is always possible that, in a dry mixture, some part of the food, possibly the test substance, may have sunk to the bottom of the pot and so have not been eaten. Taking out the remnants to weigh them and mixing them with a fresh portion of food gives the rat a second chance of eating it. It is advisable when a substance has to be tested in this way to give a rat only about 2g. in excess of what he is likely to eat from day to day, and also to make only 125g. of the mixture (100g. basal diet+25g. test substance) at a time and to use all of that before using a second small batch. Each day the bulk should be well stirred to mix again evenly before use. It is

even advisable to keep a separate batch for each rat to ensure his having received, finally, the full amount of test substance that the records of his food consumed indicate.

The daily or half-weekly dose of a liver oil or of the International Standard, both suitably diluted with the same oil, may be given as one drop from a small dropping tube or pipette. Short glass tubes 3″ long and ¼″ internal diameter fitted at one end to a narrower tube 1″ long and 0·075″ internal diameter are suitable for the purpose. A convenient practice is to make two or three dozen of these and then select for use only those which deliver, say, not more than 20·5mg. and not less than 19·5mg. of the solution. The dose is then given by letting fall one drop (or more if required) of the solution directly into the rat's mouth from the tube held upright. Greater accuracy can be attained by the use of a micrometer syringe of the Agla type fitted with a blunt injection needle. The end of this is held in the rat's mouth by one worker while the other turns the screw until the required amount has been delivered.

Sometimes the substance to be tested is a solid and so cannot be dropped into the rat's mouth. The weighed portion can generally be given to the rat by putting it, a small portion at a time, into his mouth. A drop of water or inactive oil is given after each mouthful. Generally a rat will quickly swallow everything put into its mouth, but it should be put on to a flat clean table for a minute and watched to make sure that it has done so.

(*d*) *Working out the result.*—(i) If no curve of response has been constructed and the test is arranged as described on p. 19, then the result may be worked out as follows :

Example.—Suppose the following figures have been obtained in a 3-weeks' test :—

6 rats given 0·5mg. C.L.O. daily made a mean increase in weight of 2·0g.
6 rats given 1·0mg. C.L.O. daily made a mean increase in weight of 9·3g.
6 rats given 2·0mg. C.L.O. daily made a mean increase in weight of 23·4g.
6 rats given 0·5 unit Standard daily made a mean increase in weight of 7·5g.
6 rats given 1·0 unit Standard daily made a mean increase in weight of 12·2g.
6 rats given 2·0 units Standard daily made a mean increase in weight of 28·0g.

The determination of the potency of the cod liver oil would be made thus :

Each dose of the Standard has produced a greater response than the corresponding dose of the cod liver oil. Therefore the oil contains less than 1,000 International units per gram.

Also 0·5 unit of the Standard has produced less response than 1·0mg. C.L.O. and 1·0 unit of the Standard has produced less response than 2·0mg. C.L.O. Therefore, the oil contains more than 500 International units per gram. It appears to contain about 800 units per gram. This result will probably be within about 20% of the true value. (For a discussion of probable errors, see Part II.)

The figures used for this example are more regular than are often found when small numbers of animals (*e.g.* 6) are used in a group. Even when less regular results are obtained, comparisons drawn between doses of oil and doses of Standard may be balanced so that a very fair idea of the value of the oil may be obtained.

(ii) If a curve of response has been constructed and the test has been arranged as described on p. 19, the result is worked out as follows :

If the two groups of animals which have been given doses of the substance to be tested and of the Standard respectively are found to have made equal mean increases in weight, then the determination of the vitamin A potency in terms of the International Standard is a simple proportion sum. If the mean increases in weight of the two groups are not equal, then the relative vitamin A potencies of the two doses (of test substance and of Standard respectively) must be calculated from the curve (or curves) of response relating mean increase in weight to dose of vitamin A given.

Example.—In a determination of the vitamin A potency of a sample of butter in the writer's laboratory the mean increase in weight of 10 rats (5 males and 5 females) in 3 weeks' dosing with 0·1g. daily of butter was 19·2g. and that of a similar group of rats dosed with 2 units daily of the Standard was 7·9g. Then the potency of 0·1g. of butter would *not* be $\frac{19{\cdot}2}{7{\cdot}9}=2{\cdot}43$ times the potency of 2 units of the Standard. The potencies of the two doses are in the ratio of the abscissæ of the curve of response corresponding to the mean increases 19·2g. and

7·9g. respectively. These abscissæ can be read directly from the curve of response drawn carefully on squared paper (Fig. 7), or they can be calculated from the equation representing the curve of response. Coward's equation for the curve of response of groups of rats (equal numbers of males and females) to doses of vitamin A for 3 weeks in her laboratory is

$y = 7{\cdot}14 + 23{\cdot}77 \log. x$ where y = the mean increase in weight of groups of rats in 3 weeks,

x = the dose of vitamin A given.

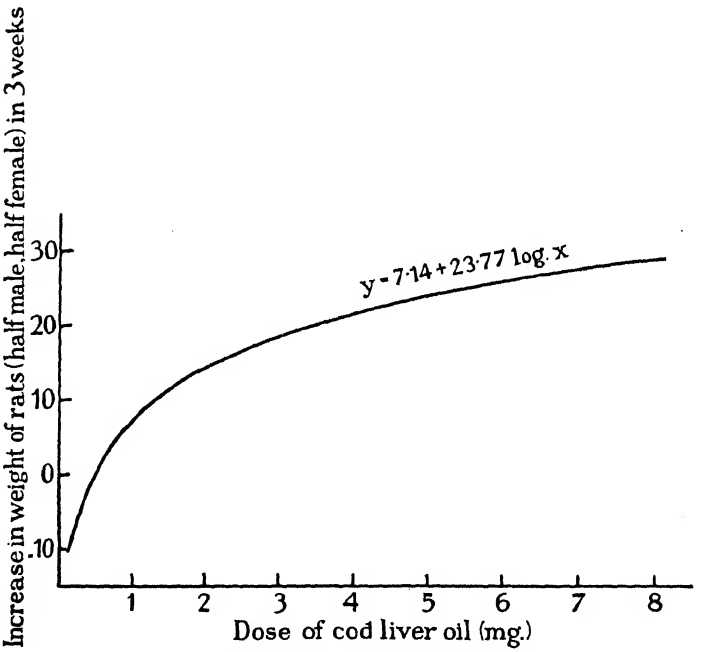

FIG. 7.—Curve of response relating increase in weight of rats (equal numbers of male and female) in 3 weeks and the dose (in mg.) of cod liver oil given daily.

Substituting for y in this equation, it is found that

when $y = 19{\cdot}2$g., $x = 3{\cdot}21$

and when $y = 7{\cdot}9$g., $x = 1{\cdot}08$.

The ratio of

$$\frac{\text{the potency of 0·1g. butter}}{\text{the potency of 2 units of the Standard}} = \frac{3{\cdot}21}{1{\cdot}08} = 3{\cdot}0$$

Therefore the potency of 0·1g. butter is 6 units and the

potency of the sample of butter is 60 International units of vitamin A per gram.

If it has not been possible to use equal numbers of males and females in both groups of rats, the results with males and females may be calculated separately from the corresponding curves of response for males and females. The curve of response for the male rats used in the construction of the curve already given is $y=6{\cdot}04+29{\cdot}62 \log. x$; that for the female rats is $y=8{\cdot}24+17{\cdot}93 \log. x$, where y and x have the same connotation as before.

Example.—A sample of fresh wheat germ was compared with a sample of cod liver oil, Z, which for some time was used as a subsidiary standard of reference for the determination of vitamin A in the writer's laboratory. Its potency had been determined as 1,500 International units of vitamin A per gram by comparison with the International Standard (avr. from nine laboratories, M.R.C. Report, No. 202, 1935). The potency of the wheat germ was determined as in Table II.

TABLE II

Dose given.	No. of animals.	Average increase in weight in 3 weeks. g.	Abscissa of curve of response corresponding to average increase in weight.	Weighted mean of abscissæ.
1·0g. wheat germ	5♂	14·4	1·91	5×1·91= 9·55
	7♀	17·4	3·24	7×3·24=22·68
				12)32·23
				2·69
2mg. C.L.O. Z	3♂	8·7	1·23	3×1·23= 3·69
	7♀	11·7	1·56	7×1·56=10·92
				10)14·61
				1·46

Then the ratio of

$$\frac{\text{the vitamin A potency of 1·0g. wheat germ}}{\text{the vitamin A potency of 2mg. C.L.O. Z}} = \frac{2{\cdot}69}{1{\cdot}46}$$

Since 2mg. C.L.O. Z contain 3·0 International units of vitamin A, then 1·0g. wheat germ contains $\frac{2{\cdot}69}{1{\cdot}46} \times 3 = 5{\cdot}5$ International units of vitamin A.

β. The "Preventive" Method

In this method the rats are given doses of test substance or of Standard from the beginning of the experiment, though some workers give their rats a preparatory period of 2 (or even more) weeks during which no supplement is given to any of the rats. Thus the rats' reserves of vitamin A are not exhausted when the doses are first given and the success of the experiment depends on getting different average increases in weight in the two groups of rats given different doses of the Standard and in the two groups of rats given different doses of the test substance.

(*a*) *The test period.*—It is advisable to have five groups of not less than 6 rats in each group for a prophylactic test. Each group must contain the same number of rats, one or two, from each litter used, so that each litter is represented equally in each group. This is important as there is no treatment for reducing all the rats to a similar state of vitamin depletion as there is when a preparatory period of feeding on the vitamin-deficient diet is allowed. Each group should contain equal numbers of males and females. The rats may be 20–50g. in weight according to their reserves of vitamin A which can only be inferred from a general knowledge of the behaviour of the rats of the colony when given a vitamin A-free diet.

The doses of the Standard should be in the ratio 2 : 1 and the doses of the test substance also, if some information of the potency of the substance is already available. If not, then a preliminary test is made with doses in a greater ratio, say, 5 : 1, or, if preferred, several groups of rats may be given doses of the test substance in the ratio 8 : 4 : 2 : 1.

The doses are given in the same way as in a "curative" test, each rat being kept in a separate cage for the whole of the period during which doses are given. One week's dose may be given in two half-weekly doses instead of one-seventh of the amount daily. The rats are weighed once a week for about the first 4 weeks, then oftener when differences may

begin to show. The test is carried on until the control rats given no supplement have died and until there are distinct differences in the increases in weight of the groups of rats given graded doses of both the test substance and the Standard.

All the rats should be kept on test for the same length of time, even if the different litters have been started at different times.

(*b*) *Working out the result.*—A " composite curve of growth " should be constructed for each group of rats. To do this, the average weights of the rats of each group at the beginning of the experiment and at the end of each week afterwards are calculated. The averages so obtained are plotted against the time and the slopes of the curves so constructed may be compared. The potency of the test substance is obtained by comparing the effects of different doses of test substance with those of the different doses of Standard as was demonstrated in the example on p. 32.

B. Occurrence of xerophthalmia as a criterion for the determination of vitamin A.

α. Curative Method

The condition which sometimes develops in the eyes of rats which are fed long enough on a vitamin A deficient diet to exhaust their reserves of that factor has been variously named ophthalmia, xerophthalmia and keratomalacia. The eyelids first become bare and swollen. Then they bleed and form an exudate which may make it impossible for the rat to open its eyes. The eyeball itself becomes affected, a mass of pus may form in it and the sight of the eye may be completely lost; if it is, it cannot be recovered. If, however, vitamin A is given in the early stages of the condition recovery is complete.

The difficulty of using the onset of ophthalmia as a sign that the rat's vitamin A reserves are exhausted and that it is therefore ready to be used in an experiment for the determination of vitamin A in any substance, is to decide on the degree of severity of this condition which it is useful to take as the criterion. Certainly it should be some stage before the eye itself is attacked; a good stage to take is that where bleeding has just begun. It probably matters very little which stage is taken, provided that the worker or workers on any one experiment can recognise and use the same stage.

(*a*) *The preparatory period.*—Rats such as those used in the "increase in weight" method are suitable for this also. Similar laboratory conditions of housing and diet may be used. The weighing of the rats is also advisable, even though it is not to be used as a criterion of the experiment.

(*b*) *The test period.*—The same methods of dosing may be used as in the "increase in weight" method. The measurement to be made is the time taken to cure the ophthalmia developed in the preparatory period. Here another difficulty presents itself, the difficulty of deciding on which day each rat may be considered to be cured, but again some agreement can be made between the workers on any one experiment so that the error of diagnosis of cure may be as small as possible.

It may be possible to construct a curve of response relating the time taken to effect a cure and the dose of vitamin A given. The writer is not aware that anyone has so far constructed one. In the absence of such a curve the test would have to be arranged as in the "increase in weight" method when no curve of response has been constructed.

There should be four or five groups of animals, two of which are given graded doses of the International Standard and the others graded doses of the substance to be examined. There seems to be a general opinion that four or five times as much vitamin A is required to cure ophthalmia as is required to bring about an increase in weight in rats which have ceased to grow on a diet deficient in vitamin A.

(*c*) *Working out the result.*—The number of days taken to effect a cure by the rats of each group are averaged and the averages compared as in the example of the "increase in weight" method on p. 32.

β. Preventive Method

The experiment to be carried out by this method is arranged exactly like the "prophylactic" "increase in weight" method, the measurement taken being the number of days which elapse before the development of the desired stage of ophthalmia. The result is worked out in a similar way.

C. Changes in the vaginal contents as a criterion for the determination of vitamin A.

The normal œstrous cycle of a rat extends over a period of 5–6 days. It may be described briefly as a period of about

3 days (dioestrus) when the vaginal contents consist of millions of leucocytes and a few nucleated cells with a little mucus, followed by a period of about 6 hours when there are no leucocytes but thousands of nucleated cells, then a period of about 24 hours when the nucleated cells are mixed with about equal numbers of keratinised cells (" cornified " cells), and then a period of 24 hours when the whole of the contents of the vagina appear to consist of these keratinised cells. Leucocytes then reappear, at first mixed with the keratinised cells, and later alone (Fig. 3). It is normally a consequence of ovulation and does not occur in ovariectomised rats (Long and Evans, 1922).

When rats are fed on a diet deficient in vitamin A and their reserves of that factor are becoming exhausted, keratinised cells are found in the vagina day after day whether they have been ovariectomised or not. If they are then given vitamin A the keratinised cells in time disappear, and in uncastrated animals normal cycles reappear (Evans and Bishop, 1922, and many later investigators, amongst whom may be mentioned Hohlweg and Dohrn (1930) who suggested the term " Kolpokeratose " for this condition).

The difficulty of applying this method to the determination of vitamin A is that, although the response of the rat to large doses of that factor is well marked, it is difficult to estimate small responses to small doses. Some definite criterion has to be decided upon so that measurements may be made on individual animals and the measurements for the group averaged. An early attempt by the writer to use this for the determination of vitamin A by means of young rats failed, for many of them became steady in weight before the vagina opened. With older rats, however, the method was more successful.

It has been suggested by Baumann and Steenbock (1932) that this criterion might be used for the determination of vitamin A by the use of mature rats and that the same rats might possibly be used over and over again.

Moll, Dalmer, Dobeneck, Domagh and Laquer (1933) used this symptom of vitamin A deficiency as the basis of a method for determining the factor in cod liver oil and foodstuffs. By an ingenious graphical representation of the course of the cure and its duration, they measured the effect of a single dose on

each animal and averaged results from animals given the same dose. They decided that each rat's behaviour in its first use for the test was more erratic than its behaviour to subsequent doses and the first result with any one rat should therefore be discarded. Even when this precaution was observed, they decided that the accuracy of the test was not greater than that obtained by the "increase in weight" method, but that, being specific for vitamin A, it made a good biological control of the "increase in weight" method.

The following method was worked out by Coward, Cambden and Lee (1935):

(*a*) *Preparatory period.*—Rats of about 140g. weight or more which have grown up on a diet only moderately rich in vitamin A are used. Smears of the vaginal contents are examined daily, a small spatula being inserted in the vagina, pressed against the wall and withdrawn with some of the contents of the vagina adhering. This material is rubbed off on to a small drop of water on a microscope slide, at once examined under the low power of the microscope and a diagnosis made. No staining is necessary. Thus if the first smear proves unsatisfactory a second can be made immediately. When it is established that the rats are having cycles regularly and are therefore probably healthy animals, they are given a vitamin A-deficient diet until keratinised epithelial cells are found in the vagina for 10 successive days. They are then considered ready for the test.

(*b*) *The construction of a curve of response relating the number of days elapsing before the disappearance of keratinised cells from the vagina, and dose of vitamin A given.*—As the rats become ready for test, the first 7 are given in turn one dose only of 5, 10, 20, 40, 60, 100 and 200mg. respectively of a certain sample of cod liver oil. The next 7 rats are given similar doses in turn also, and the next until 10 rats have received 5mg., 10 have received 10mg. and so on. Vaginal smears are examined daily, Sundays included, and the number of days elapsing before the keratinised cells disappear from the vagina is taken as the measurement required for the test. The periods required by the different rats given any one dose are averaged and the averages from the different groups of rats are plotted against the dose of cod liver oil given. The lower doses may be insufficient to bring about a positive reaction in

all the rats to which they were given. Coward found that if a rat did not respond in 10 days it never did, so for the purpose of averaging the results from a group of rats each rat which did not respond in 10 days was given the value 10. With this convention, a fairly smooth curve of response was obtained (Fig. 8). It was logarithmic in shape and was represented by the equation $y=13{\cdot}2-4{\cdot}5 \log. x$, where y=the number of days elapsing between the giving of the dose and the disappearance of keratinised cells from the vagina, and x was the single dose (mg.) of cod liver oil given.

The curve of response is used as the curve relating the

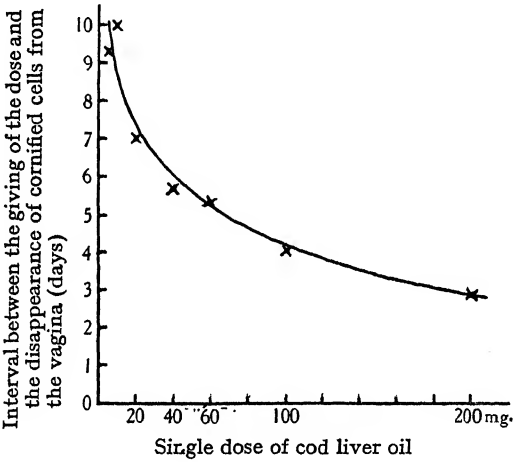

FIG. 8.—Curve of response relating the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

increase in weight of rats to doses of vitamin A given is used. Two groups of rats are prepared as described above, each rat of one group then being given a dose of the Standard and each rat of the other group a dose of the test substance. The average time elapsing in the two groups between the giving of the dose and the disappearance of keratinised cells from the vagina is determined. The relative potency of the dose of test substance and dose of Standard is given by the ratio of the abscissæ corresponding to the average results (days) of the two groups.

If no curve of response has been constructed, then the test

can be arranged as it is in the " increase in weight " method when no curve has been constructed.

This method of determining vitamin A might be made more accurate by ovariectomising all the rats when the influence of ovulation would be removed.

(*c*) *The duration of the " cure " from a single dose of vitamin A.*—Smears from the rats in the construction of Coward's curve of response to a single dose of cod liver oil were examined daily until keratinised cells again appeared continuously in the vaginal contents, 10 days' continuous occurrence of these cells being the criterion adopted as evidence of vitamin A

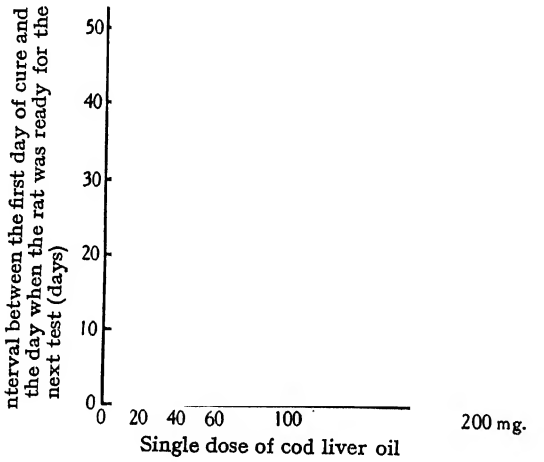

FIG. 9.—Curve of response relating the dose of vitamin A to the ' duration of the cure " of the condition of the vaginal contents.

deficiency. A curve of response for the " duration of cure " was obtained, curvilinear in shape but not logarithmic (Fig. 9). It showed that the size of the dose of vitamin A given influences not only the rapidity of the response but also the duration of the rat's resistance to a subsequent shortage of vitamin A.

(*d*) *The possibility of using the same rats for several successive tests.*—Each rat in Coward's experiment which had responded to any given dose of cod liver oil was given no further dose of vitamin A until it had again reached the stage when it was considered ready for the test, *i.e.* when it had again shown keratinised cells in the vagina for 10 successive days. It was then given a dose of the same cod liver oil equal to the dose

first given and the response to this second dose compared with the response to the first dose. It was found with all the doses that the rats took longer to respond to the second dose than to the first dose. They had become less sensitive to their doses of cod liver oil. Hence rats which are used a second time for this test must only be used for comparison with other rats which are being used a second time.

The accuracy obtainable by the method of determination was found to be less than that obtainable in the 3 weeks' " increase in weight " method.

D. **Comparison between prophylactic and curative methods.**

As only one " end-point " is needed in the prophylactic method, it would appear that this method had a great advantage over the curative method which involves two end-points, one at the beginning of the curative period and the other at the end. It is also claimed as an advantage of this method that the rats do not have to bear the strain of the period when their reserves of vitamin A are nearly exhausted before they are given a dose of test substance or Standard for the curative part of the test.

Against the first argument in favour of the prophylactic method is the fact that individual rats vary so much in the amount of their vitamin A reserves that more rats would have to be used in a comparison of unknown and standard than would have to be used in a curative test where the rats are brought to a more or less similar state of depletion by the preliminary feeding on the vitamin A-free diet. That the rats are in a varied state of ill-health after their depletion period is evident from the fact that when given a small dose of vitamin A some rats continue to lose weight and die, whereas others recover and even grow slowly. If similar rats are given a larger dose of vitamin A, possibly one or two may die, but the majority will grow, though at different rates. The surprising point about this variation in response to doses of vitamin A is that, among the rats which respond and grow, it is no greater than the variation in growth of rats of similar age or weight in a normal colony. This was shown by Coward (1932) in an examination of over 2,000 experimental rats and nearly 500 normal rats from the same colony. Thus the only argument

brought forward in favour of the prophylactic test seems to be based on a wrong assumption. The advantages of the curative method are numerous. It takes a shorter time and, if many tests are to be performed, a curve of response can be used to save labour and expense. The advantages of the curative method over the prophylactic method seem therefore to be incontrovertible.

E. Comparison between (a) the "increase in weight" method, (b) the "xerophthalmia" method and (c) the "vaginal contents" method.

The "increase in weight" method has the distinct advantage over the other methods in having a criterion that is easily measured, though even this is not quite as accurate as one might at first imagine it to be, for a rat's weight fluctuates during 24 hours—it does not increase or decrease at a steady rate. This criterion is, however, much more accurately measured than either of the other criteria used for the determination of vitamin A, the error due to the uncertainty of whether xerophthalmia may be considered cured or not on any particular day being much greater than the error due to the fluctuation in weight of a rat during the day.

On the other hand, both the occurrence of xerophthalmia and the changes in the vaginal contents are thought to be due specifically to vitamin A deficiency and many people hold the opinion that a determination based on a reaction which is specific for the factor being measured is far better than a more accurate method based on a reaction such as increase in weight which is not specific for any one substance.

4. Physical Properties of Vitamin A by means of which it may be Measured

Vitamin A, as it occurs in liver oils and concentrates, shows an apparently characteristic absorption band in the ultra-violet region with a maximum at 328mμ.

The blue colour which is produced when a liver oil or concentrate is treated with arsenic or antimony trichloride is probably due to the formation of a derivative of vitamin A.

Either of these properties could be used for the measure-

ment of vitamin A if it were certain (*a*) that no other substances acted in a similar manner and thus enhanced the measurement, and (*b*) that no other substances interfered with the reaction and diminished the measurement. A great deal of work has been done to try to find ways of eliminating these possibilities, but the difficulty is not yet wholly solved.

(A) *The absorption of rays of wave-length* 328mμ.—In the earlier work the measurement of absorption at 328mμ was made on the oil itself. Then it was shown that the presence of unsaturated fatty acids raised the value obtained. Even blowing air or oxygen through the oil could quadruple the value. It therefore became obvious that the measurement should be made on the unsaponifiable part of the oil and not on the oil itself. It also became evident that different results were obtained when different solvents were used in the determination. A small subcommittee was appointed by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute to investigate the methods already in use for measuring the intensity of absorption at 328mμ by oils and their concentrates, and to make recommendations for the guidance of other workers in this subject. The members of the subcommittee examined six different samples of cod liver oil and one concentrate, measuring the intensity of absorption at 328mμ of the oil itself and also of the unsaponifiable part of each oil, prepared in different ways. The cod liver oils chosen included medicinal oils, oils intended for cattle or poultry feeding, one crude oil and one " coast cod " oil. The results obtained on these oils have been published in some detail in a Special Report, No. 202, of the Medical Research Council. The conclusions reached by the members of the subcommittee who examined these oils were summarised thus :

(i) Estimations on cod liver oils should be made on the unsaponifiable fraction of the oil.

(ii) Cyclohexane or ethyl alcohol and not chloroform should be used as solvent.

(iii) Only a method of saponification approved for the purpose should be used.

The method of saponification selected by the International Conference on Vitamin Standardisation, 1934, is the following :

One gram of oil is saponified with 10cc. N/2 freshly prepared alcoholic KOH, by boiling until clear (time needed about

5 minutes). 20cc. water are added, the whole transferred to a small separator and extracted with two quantities of 25cc. ether (peroxide-free). The ethereal extracts are washed first with water (10–20cc.), then with 10–20cc. N/2 KOH and again with water, while rotating gently without shaking. The ethereal solution is then shaken thoroughly with two quantities of 10cc. water, after which it is filtered into a flask, the ether evaporated almost to dryness and the residue dissolved in ethyl alcohol or cyclohexane and made up to the concentration required for the particular instrument in use. A preliminary test on the original oil will indicate the amount both of oil and of solvent which will be necessary.

Pure cyclohexane, suitable for spectrographic examination, should have the following properties: $d^{20°}_{4°}=0{\cdot}7784$; B.P. 81·4° C.; F.P. 6·5° C.; it should be almost completely transparent in the region of 328mμ and exhibit no trace of discontinuous absorption.

Conversion of values found spectroscopically into International units of vitamin A activity.—The intensity of absorption of an oil at 328mμ is expressed in the form $E^{1\%}_{1cm.}=x$. That is, the value must be stated as for a 1% solution of the oil (or the corresponding amount of unsaponifiable matter) in alcohol or cyclohexane, and the solution must be examined in a cell of 1cm. thickness. E is the log. of the ratio between I_0, the intensity of the incident light and I, the intensity of the emergent light. Thus, suppose the value of $E^{1\%}_{1cm.}$ for a particular oil is given as 1. This means that only 10% of the incident light is transmitted by a depth of 1cm. of a 1% solution of that oil; for, given log. $I_0/I=1$, therefore (since log. $10=1$) the intensity of the incident light is ten times the intensity of the emergent light; or the emergent light is 10% of the incident light and a depth of 1cm. of a 1% solution of the oil has absorbed 90% of the incident light. Similarly, if the value of $E^{1\%}_{1cm.}$ were given as 1·3, then log. $I_0/I=1{\cdot}3$ and, since log. $20=1{\cdot}3$, the intensity of the incident light is twenty times the intensity of the emergent light; thus the emergent light is only 5% of the incident light, and a depth of 1cm. of a 1% solution of the oil has absorbed 95% of the incident light.

If a few more values are substituted for $E^{1\%}_{1cm.}$ and the corresponding percentages of light absorbed are determined and all the percentages so obtained are plotted against the

corresponding values of $E^{1\%}_{1cm.}$, it is found that the relationship is curvilinear. This may appear disturbing until it is realised that the percentage of light absorbed is not proportional simply to the concentration of the absorbing substance. That the values for $E^{1\%}_{1cm.}$ are proportional to the concentration of vitamin A in a solution (provided no interfering substances are present) may be deduced from the general formula

$$E = \log. I_0/I = ecd, \qquad \text{where } e \text{ is a constant}$$

for the substance under examination, c is the concentration and d is the thickness of the layer of solution examined. In the measurement of vitamin A, e is a constant, d is always 1cm. and therefore constant; therefore $E^{1\%}_{1cm.} = Kc$, that is $E^{1\%}_{1cm.} \propto c$ or $E^{1\%}_{1cm.}$ varies directly as c. Thus the measure, $E^{1\%}_{1cm.}$, may be taken as a measure of the vitamin A content of liver concentrates.

The Permanent Commission on Biological Standardisation adopted provisionally the factor 1600 for converting the E value of an oil into the biological value in terms of the International unit. The evidence on which the decision to do this was based is summarised in the Special Report, No. 202, of the Medical Research Council. There has been some dispute as to the validity of this factor, and the matter is under consideration. An early report (Hume, 1937) of an extensive investigation of a halibut liver oil shows that there is, so far, no good evidence that the factor should be changed.

(B) *The "Blue Value" of an oil or concentrate.*—It seems to be agreed that an oil which gives no blue colour when treated with arsenic trichloride or antimony trichloride, contains no vitamin A, and that an oil which gives a deep blue colour is rich in vitamin A. Various workers have tried to decide how far the measurement of the blue colour was quantitative and, also, how close was the correlation between the blue colour and the vitamin A value of an oil as measured biologically. Crude methods of measuring both the blue value and the biological value at first gave a fair amount of agreement. Later, as the methods of making both measurements became more accurate, and in particular, as the "error" of the biological method became estimable, it became obvious that the correlation between the two values was not good. Indeed, one worker showed that there might be as much as 400% difference between

the two. It was, however, shown that if the " blue value " of the unsaponifiable fraction of an oil were measured instead of that of the oil itself, a better correlation was found between this value and the vitamin A value of the oil measured biologically.

A more careful examination of the blue colours obtained in the reaction with different oils has revealed differences which may account for the poor correlation between the blue values and the biological (vitamin A) values of the oils. Morton, Heilbron and Thompson (1931) and Heilbron, Gillam and Morton (1931) showed that when examined spectroscopically the blue pigment had two strong absorption bands with maxima at 606 and 572mμ respectively. In concentrates, the 606mμ band was shifted to 617mμ and the 572mμ band to 583mμ. The two bands were not always of the same relative intensity in different oils; either might be the stronger. They showed also that the intensity of the 606mμ band could easily be increased by treating the oil with hydrogen peroxide or by blowing air through it, though prolonged treatment of this kind ultimately destroyed both the 606mμ and the 572mμ bands. They concluded that the substance which gave the 572mμ band was vitamin A, and Morton (1932) proceeded to develop a method first suggested by Emmerie, Eekelen and Wolff (1931) by means of which the blue colour measured by the 606mμ band could be suppressed, so that only the colour giving the 572mμ band would be measured as the " blue value " of the oil. Morton found that the addition of a trace of 7-methyl-indole to the solution of oil or concentrate before the addition of the antimony trichloride solution inhibited the development of the 606mμ (or 617mμ) band. The addition of a larger amount inhibited the development of both bands, 572mμ and 606mμ (583mμ and 617mμ in concentrates), but if just enough were added to reduce the 606mμ band to the intensity of the 572mμ, then the potency as determined by the intensity of the 572mμ band was in close agreement with the potency as determined by the 328mμ band.

The significance of these absorption bands became even more apparent in the work of Heilbron, Heslop, Morton, Webster, Rea and Drummond (1932) on the preparation of nearly pure vitamin A. From an exceedingly rich source of vitamin A (halibut liver oil), these workers extracted the

unsaponifiable fraction and by distillation in very high vacua prepared concentrates which consisted of very nearly pure vitamin A. The work of Karrer, Morf and Schopp (1931, 1, 2) was on very similar lines. Spectroscopic examination of the " blue colour " of the various fractions obtained during the concentration showed the bands 617mμ and 583mμ in relatively the same intensity throughout the process. Moreover, an additional band at 695mμ was also seen in the blue colour of each of the fractions of the same intensity relative to the other two bands throughout the concentration. Similar concentrates from sturgeon liver oil (another very rich source of vitamin A) showed the same band at 695mμ, only its intensity relative to the other bands was different from that found in halibut liver oil concentrates. In concentrates from mammalian liver oils, the band was entirely absent. These results convinced the workers that they had not obtained pure vitamin A.

The results of the whole of this work indicate very strongly that a simple measure of the whole " blue value " of an oil or its concentrate cannot be an accurate measure of vitamin A.

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CHAPTER IV

THE DETERMINATION OF VITAMIN B_1

1. The International Standard of Reference and the Unit of Vitamin B_1 Activity.
   - A. The treatment of the Standard of reference before giving it to the animals.
   - B. The need for a simultaneous test of the Standard of reference whenever a determination of vitamin B_1 is made.
   - C. The general arrangement of the test for a determination of the vitamin B_1 potency of a substance in terms of the International Standard.
2. The Preparation of Pigeons for a Vitamin B_1 Determination.
   - A. Pigeons suitable for the test.
   - B. Housing of the pigeons.
   - C. Vitamin B_1-free diet.
3. The Preparation of Rats for a Vitamin B_1 Determination.
   - A. Rats suitable for the test.
   - B. Housing of the rats.
   - C. Vitamin B_1-free diet.
4. Criteria for the Measurement of the Response of Animals to Doses of Vitamin B_1.
   - A. Cure of retracted neck in pigeons.
   - B. Increase in weight in rats.
   - C. Cure of convulsions in rats.
   - D. Cure of bradycardia in rats.
   - E. Comparison of the four methods.
5. References.

VITAMIN B_1 is the name adopted by the Accessory Food Factors Committee of the Lister Institute and Medical Research Council and by the Biochemical Society for the factor which is essentially the antineuritic factor. It prevents and cures beri-beri in man, polyneuritis in pigeons and rats and is essential for the growth of the rat. It is called vitamin B by some workers in the United States of America. It has been prepared in the crystalline state as hydrochloride and sulphate by several workers and synthesised by three different methods, but the original adsorption product from an aqueous extract of rice polishings has proved very satisfactory and is, for the present, retained as the International Standard of reference. When

the supplies of the adsorption product are exhausted a sample of the pure substance will probably take its place.

1. The International Standard of Reference and the Unit of Vitamin B_1 Activity

The Standard of reference for vitamin B_1 which was recommended in 1934 by the Permanent Commission for Biological Standardisation of the Health Organisation of the League of Nations was the same as the one adopted in 1931 by the same Commission as a provisional standard of reference. It is a particular sample of an adsorption product on fuller's earth of an extract of rice polishings. It was prepared by Jansen in the Medical Laboratory, Batavia, Java, by the method of Seidell. It is kept at the National Institute for Medical Research, London. It has proved to be stable for the last five years. No special precautions are necessary for preserving its activity except that it should be stored in a dry place. In the presence of moisture bacterial decomposition readily sets in.

The International unit of vitamin B_1 activity is the amount of activity contained in 0·01g. of this particular preparation.

A. The treatment of the Standard of reference before giving it to the animals.

The International Standard of reference is a dry whitish powder, insoluble in water. The dose which cures about 50% of pigeons with retracted neck is 0·03g., *i.e.* 3 units. To give this dose to a pigeon the amount is weighed on a watch-glass and mixed with about 2ml. distilled water. This is taken up in a pipette to which a length of about 2″ of rubber tubing is attached and, by passing the free end of the tubing about 2″ down the throat of the pigeon it is delivered directly into its crop. The residues on the watch-glass are taken up with a little more water into the pipette and given to the pigeon in the same way.

Doses of 0·01g. (1 unit) and 0·02g. (2 units) have been found to give useful results with rats, *i.e.* moderate responses. These amounts are conveniently weighed on little porcelain paint dishes, one for each rat. The dose is moistened with water and put in the rat's cage. The rats take their doses of this pre-

paration so readily that there can be no loss of activity during the short time that the vitamin B_1 is standing in contact with the water.

B. **The need for a simultaneous test of the Standard of reference whenever a determination of vitamin B_1 is made.**

There are no records in the literature of one particular substance having been examined repeatedly to see how far the results of successive determinations of its vitamin B_1 content might vary, even when the conditions of the tests were thought to be the same. During the last five years, however, the writer has used the International Standard of reference for determining the vitamin B_1 potency of different substances, which has always involved a test on the Standard simultaneously with each determination made. Twenty-one tests on the Standard have thus been made, the same dose 0·03g. (3 units) being used in all the tests. The results may be seen in Table III.

TABLE III

THE VARIATION IN THE PERCENTAGE NUMBER OF PIGEONS IN DIFFERENT GROUPS CURED OF RETRACTED NECK BY A SINGLE DOSE, 0·03G. (THREE UNITS) OF THE INTERNATIONAL STANDARD

	Date of test.	No. of pigeons given 0·03g. of the Standard.	No. of pigeons cured.	Percentage of pigeons cured.
1	October, 1932	23	11	47·8
2	October–November, 1932	15	7	46·7
3	January–February, 1934	16	8	50·0
4	June–September, 1934	11	6	54·5
5	October–December, 1934	14	9	64·3
6	February, 1935	9	3	33·3
7	April–May, 1935	9	6	66·7
8	July, 1935	8	6	75·0
9	September, 1935	8	4	50·0
10	January–February, 1936	9	6	66·7
11	April, 1936	9	6	66·7
12	May, 1936	7	4	57·1
13	May, 1936	9	4	44·4
14	July, 1936	9	5	55·6
15	August, 1936	12	8	66·7
16	September, 1936	7	6	85·7
17	October, 1936	9	7	77·8
18	November, 1936	10	7	70·0
19	December, 1936	7	3	42·9
20	January, 1937	5	3	60·0
21	March, 1937	10	6	60·0

As the percentage of birds cured by the same dose, 3 units of vitamin B_1, varies, it is evident that a simultaneous test of the Standard must be made whenever the vitamin B_1 potency of a substance is to be determined.

Fluctuations in the average duration of the cure of retracted neck in pigeons are also found (Table IV).

TABLE IV

THE VARIATION IN THE AVERAGE DURATION OF CURE OF RETRACTED NECK IN DIFFERENT GROUPS OF PIGEONS CURED BY A SINGLE DOSE OF 0·03G. (THREE UNITS) OF THE INTERNATIONAL STANDARD

	Date.	No. of birds cured.	Average duration of cure (days).
1	October, 1932	11	3·7
2	October–November, 1932	7	2·1
3	January–February, 1934	8	3·5
4	June–September, 1934	6	4·5
5	October–December, 1934	9	5·3
6	February, 1935	3	3·3
7	April–May, 1935	6	3·2
8	July, 1935	6	4·7
9	September, 1935	4	4·2
10	January–February, 1936	6	3·3
11	April, 1936	6	4·3
12	May, 1936	4	2·8
13	May, 1936	4	5·0
14	July, 1936	5	7·2
15	August, 1936	8	6·2
16	September, 1936	6	7·3
17	October, 1936	7	6·4
18	November, 1936	7	4·7
19	December, 1936	3	6·0
20	January, 1937	3	10·7
21	March, 1937	6	4·2

As the average duration of the cure of retracted neck in different groups of pigeons (each pigeon having been given 3 units of vitamin B_1) varies, it is evident that a simultaneous test of the Standard must be made whenever the vitamin B_1 potency of a substance is to be determined.

It has been suggested by Lassen (1936) that the average " duration of cure " of the birds should include the value 0 for those birds which were not cured, but this cannot be considered a sound procedure since there can be no value in this record of response less than 0 and any value recorded as 0 might have

really been less than 0, if it had been possible so to record it. Thus the only way to use the criterion " duration of cure " is to use it as its name implies, the duration of the cure effected by doses of vitamin B_1.

Fluctuations in the average increase in weight of groups of rats in response to doses of the International Standard are also found. Table V gives results obtained during the last three years in the writer's laboratory.

The average increase in weight in 3 weeks from 4 or more rats given 0·01g. (1 unit) of the International Standard varied from −9·5g. to +15·6g., and from 4 or more rats given 0·02g. (2 units) of the International Standard it varied from 8·5g. to 33·85g. The groups of animals (4 or more) are certainly small, but Coward, Burn, Ling and Morgan (1933) have shown that a smaller number of rats is required for a given degree of accuracy in a vitamin B_1 determination than in a vitamin A determination. It is obvious from these figures that a simultaneous test of the Standard of reference must always be made whenever the vitamin B_1 potency of a substance is to be determined.

C. The general arrangement of the test for a determination of the vitamin B_1 potency of a substance in terms of the International Standard.

The same principles must guide the general arrangement of the test for a vitamin B_1 determination as for a vitamin A determination. It must be the object of the experimenter to find a dose of the substance examined which gives a response equal to that given by a dose of the Standard examined at the same time on similar animals. The responses to be aimed at are submaximal and preferably about half-way between no response and " complete " response. Thus whether pigeons or rats are the test animals, if no information of the probable potency of the test substance is available, the animals as they become ready for the test should be distributed in turn into, say, five different groups, two of the groups being given doses of the International Standard in the ratio 2 : 1 and the other three groups being given doses of the substance to be examined in a very wide range of doses, say, 9 : 3 : 1. This preliminary test may be made on 4 or 5 pigeons in each group of a pigeon test, or 3 or 4 rats in each group of a rat test, but it should always be followed by a further test on, say, three or four groups

TABLE V

THE VARIATION IN THE RESPONSE OF RATS TO DAILY DOSES OF THE INTERNATIONAL STANDARD

Date of test	Daily dose, 0·01g. International Standard.					Daily dose, 0·02g. International Standard.				
	No. of ♂'s.	Average increase in weight in 3 weeks, g.	No. of ♀'s.	Average increase in weight in 3 weeks, g.	Average increase calculated as if from equal Nos. of bucks and does, g.	No. of ♂'s.	Average increase in weight in 3 weeks, g.	No. of ♀'s.	Average increase in weight in 3 weeks, g.	Average increase calculated as if from equal Nos. of bucks and does, g.
April, 1933	2	−17·0	2	−2·0	−9·5	2	36·0	2	24·0	30·0
August, 1933	2	5·5	2	7·0	6·25	1	29·0	3	23·7	26·35
October, 1933	—	—	—	—	—	3	15·0	1	2·0	8·5
October, 1934	2	2·0	2	−6·5	−2·25	1	20·0	3	13·0	16·5
October, 1934	2	23·5	3	3·7	13·6	4	21·7	2	26·0	23·85
November, 1934	2	9·0	3	−1·0	4·0	3	21·0	2	17·5	19·25
June, 1935	3	9·0	2	6·5	7·75	3	22·0	2	24·5	23·25
July, 1935	3	1·7	4	3·5	2·6	4	30·5	3	22·7	26·6
October, 1935	2	7·0	2	15·5	11·25	2	26·0	3	23·0	24·5
November, 1935	3	−1·3	1	−3·0	−2·15	3	18·0	1	12·0	15·0
November, 1935	3	−4·3	2	0·3	−2·0	3	17·3	2	14·5	15·9
December, 1935	4	3·0	1	19·0	11·0	4	23·0	1	14·0	18·5
February, 1936	3	8·7	2	7·0	7·85	4	19·7	1	24·0	21·85
February, 1936	3	11·0	3	5·0	8·0	3	23·0	3	23·0	23·0
May, 1936	4	15·2	2	16·0	15·6	3	36·7	2	31·0	33·85
June, 1936	3	7·7	3	−4·0	1·85	3	18·0	3	23·7	20·85
July, 1936	4	5·8	3	3·7	4·75	4	30·2	4	19·2	24·7
July, 1936	3	10·7	1	19·0	14·85	2	24·5	2	27·5	26·0
August, 1936	2	3·0	2	4·5	3·75	2	19·5	2	19·0	19·25
September, 1936	3	0·0	1	−10·0	−5·0	3	12·0	2	6·0	9·0
September, 1936	3	6·0	1	−1·0	2·5	3	19·3	0	—	—

of animals, two of these being given doses of the Standard in the ratio of 2 : 1 and one group being given a dose which, judging from the preliminary test, will give a result somewhere between those given by the two doses of the Standard; or two groups may be given doses of the test substance in the ratio 2 : 1 which may be expected to give results equal to those given by the two doses of the Standard.

The use of a curve of response.—If many vitamin B_1 determinations are contemplated it is exceedingly useful to construct a " curve of response " relating the percentage of birds cured to the dose of vitamin B_1 given. To do this, not less than 36 birds with retracted neck will be required. As

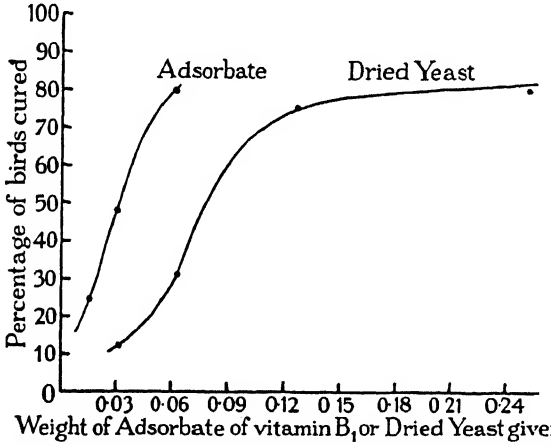

FIG. 10.—Two examples of sigmoid curves, relating percentage of birds cured to dose of vitamin B_1 given.

they become available, doses of 0·01g., 0·03g., 0·06g. are tested in turn, the first pigeon being given 0·01g., the second 0·03g., the third 0·06g., the fourth 0·01g., and fifth 0·03g., the sixth 0·06g., and so on, until each of the three doses has been tested on 12 birds. If desired, higher and lower doses than these may be tested, the doses being given in rotation with the other doses, but they will give points at the lower and upper ends of the curve which are generally considered inaccurate and not good to work upon (Fig. 10). Curves of response for " all or more " reactions such as the cure or non-cure of retracted neck in pigeons are generally sigmoid in shape. When the curve is once constructed fewer pigeons may be

used for tests. A dose of Standard must still be tested whenever a determination is made, but it is not necessary to try to determine the dose of test substance which gives the same result as the Standard. If both results (percentages of birds cured), one from the Standard and one from one dose of test substance, fall on the curve constructed from the three doses only of 0·01g., 0·03g. and 0·06g. Standard, then no further test is necessary. The abscissæ corresponding to the two "per-

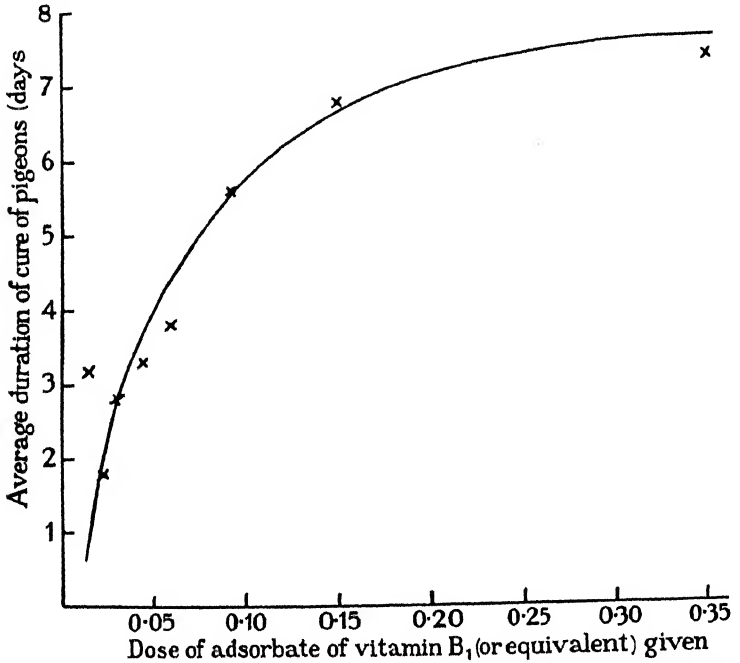

FIG. 11.—Curve of response relating duration of cure of pigeons to dose of vitamin B_1 given.

centage cures" are read from the curve of response and taken as the relative potencies of the doses of Standard and test substance respectively.

A curve of response relating the "duration of cure" (*i.e.* the number of days elapsing between the day of cure and the day on which symptoms of polyneuritis are again evident) and dose of vitamin B_1 given may also be constructed. This will probably be found to be a logarithmic curve (Fig. 11).

Similarly, in experiments on rats which have been given a

vitamin B_1-free diet until they have become steady in weight and have then been given a daily supplement, a curve of response may be constructed relating increase in weight in a given time and dose of vitamin B_1 given. Useful doses for the construction of a curve of response are 0·005, 0·01, 0·02 and 0·04g. of the International Standard. This curve of response will probably be logarithmic. Separate curves of response for male and female rats may be constructed if desired, 4 or 5

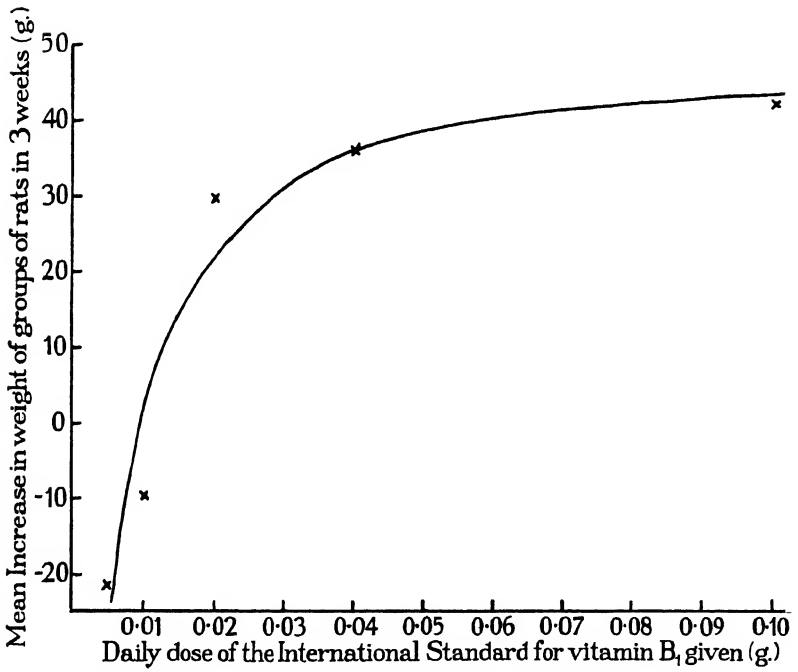

FIG. 12.—Curve of response relating increase in weight in 3 weeks to dose of vitamin B_1 (International Standard) given.

animals being used in each group, but Coward, Burn, Ling and Morgan (1933) and Coward (1936) have shown that the curve of response for male rats is only slightly steeper than that for female rats. It is more convenient to use only one curve of response and if that is constructed with equal numbers of male and female rats in each group it will be a useful curve for interpreting later results from groups consisting of equal or nearly equal numbers of male and female rats (Fig. 12). The curve is used in the same way as the curve of response relating

increase in weight to dose of vitamin A given. If some information of the probable potency of the substance is available, then one dose of the substance is tested on, say, 6 rats, and one dose of the Standard (say, 0·01g.) is tested on 6 rats. The relative potency of these doses is not the ratio of the average increases in weight of the rats given the doses, but the ratio of the abscissæ of the curve corresponding to the increases in weight. These are therefore determined and the potency of the substance under test calculated by a simple proportion sum.

It is essential that the curve of response should be constructed in the laboratory in which it is to be used and with animals from the colony which will supply the animals for the future tests. It is almost certain that no two colonies of rats would give the same curves of response, even when the basal diets were made as nearly alike as possible. It is also essential to test the curve of response to see whether it is applicable to substances other than the International Standard. Food substances of different kinds should be tested, each in two doses, one of which is double or treble of the other. The abscissæ corresponding to the increases in weight resulting from a pair of doses of each substance should be determined from the curve to see how nearly their ratio corresponds to the known ratio of the doses (see Chapter VII).

When convulsions in rats and bradycardia in pigeons are used as the criteria of vitamin B_1 deficiency, the test for the determination of vitamin B_1 can be arranged in a similar way.

2. The Preparation of Pigeons for a Vitamin B_1 Determination

A. Pigeons suitable for the test.

It is not necessary to breed pigeons specially for vitamin work. The healthy stock of any reputable dealer is suitable.

The pigeons should be 300–350g. in weight for vitamin B_1 tests. Sex is apparently immaterial.

B. Housing of the pigeons.

Cages about 3′ square by 6′ high are suitable for housing experimental pigeons. This size can accommodate about 10 birds conveniently. The cages may be arranged in series, side by side in the open air. The group of cages should be

sheltered from the wind and rain. Moderate cold does not harm the birds. Each cage should have a doorway large enough for a worker to enter the cage. It is useful to have a passage closed by wire netting in front of the whole series of cages, so that if a bird escapes from its cage it may be recaptured in the passage.

As much as possible of the structure should be of wire mesh, about 0·5″ diameter. The floor of each cage should be of wire mesh, raised about 2″ from a tray which can easily be drawn out for cleaning. This gives the birds as little access to their fæces as possible. Birds and rats fed on a vitamin B_1 deficient diet will eat their fæces in order, presumably, to obtain the vitamin which is apparently generated by bacteria but not absorbed in the lower part of the gut.

The food and water of the pigeons should be placed in troughs just outside the cages, to which access may be obtained by a narrow slit in the wall of the cage.

Even with a floor made of wire mesh some part of the pigeons' fæces will be caught, and will be obtainable by the pigeons. The walls of wire mesh will also catch droppings from the birds clinging to them. Thus the more frequently the cages are cleaned the less chance will the birds have of obtaining vitamin B_1 from their fæces and the sooner will they develop polyneuritis.

C. **Diet of the pigeons.**

Polished rice (rice with the husks and embryo removed) is still the most generally used diet for producing polyneuritis in pigeons. Some workers wash it in running water and then dry it before using it—others consider this unnecessary. The pigeons eat plenty of this at the beginning of the experiment and supplies should always be greater than their need. A plentiful supply of clean water should be given fresh every day.

3. The Preparation of Rats for a Vitamin B_1 Determination

A. **Rats suitable for the test.**

Young rats weighing 50–60g. are suitable for determining vitamin B_1 by means of its influence on the weight of the rat. Male and female rats may be used. Although Coward, Burn, Ling and Morgan (1933) and Coward (1936) found that the

response of male rats to doses of vitamin B_1 was not much greater than that of female rats, yet it is preferable to have the same relative numbers of bucks and does in the different groups which are used for comparing different doses. Thus each group in a test may contain equal numbers of bucks and does, or, alternatively, each group may contain 3 bucks and 2 does, or 2 bucks and 3 does. It is not good to have one group of a test consisting of 4 bucks and 1 doe and another group of the same test consisting of 1 buck and 4 does.

B. **Housing of the rats.**

Several rats may be kept together in one cage during the preparatory period, but during the test period each rat should be kept in a separate cage. They may be housed in the ordinary wire cages of the laboratory but with one important addition. Grids of wire mesh of $\frac{1}{2}''$ diameter standing 1" high should cover the floor of each cage. This prevents the rats from having access to their fæces. Rats on a vitamin B_1 deficient diet are specially prone to eat their fæces which may contain vitamin B_1 and thus vitiate the test. Sawdust may be used in the bottom of the cage, or sheets of blotting-paper. The grids should be examined daily and changed if found to be soiled with excreta. The same warm temperature is required for rats in vitamin B_1 tests as for those in other vitamin tests.

C. **Vitamin B_1-free diet.**

The diet of rats for tests of vitamin B_1 must contain all the substances known to be necessary for growth except vitamin B_1. A suitable diet has the following composition:

Caseinogen	15 parts
Dextrinised rice starch	79 „
Agar-agar	2 „
Salt mixture (Steenbock's 40)	4 „
Dried brewers' yeast autoclaved at 15 lb. pressure for 6 hours	25 „

In addition each rat should be given 5 or 6 drops of a good sample of cod liver oil twice a week. This should be dropped directly into his mouth, so that it is certain that each rat has received his required portion.

The caseinogen named "light white" of the British Drug Houses has been found suitable for tests on vitamin B_1. It is

not impossible that it contains small amounts of this factor, but that it does not contain appreciable amounts of it has been shown by Coward, Key and Morgan (1929). It is probably a sodium caseinate. It makes a colloidal solution in water by shaking or beating with an egg-whisk. The " Physiological Caseinate," (Glaxo Labs. Ltd.) has also been found suitable for these tests.

Dextrinised rice starch, prepared as described in the vitamin A tests, is used for the same reasons for which it is used in those tests.

Agar-agar (powdered) is included in the diet to prevent constipation which might otherwise result from a shortage of vitamin B_1.

Fresh water must be supplied each day, preferably in an inverted bottle with a narrow neck. Any water which is fit for human consumption may be used for the rats. Distilled water is not necessary.

Autoclaved yeast must be supplied plentifully in the diet. It cannot be assumed that the amount of dried yeast which has been found to be abundance in a vitamin A-free diet will, when autoclaved, supply abundance of vitamin B_2, etc., in a vitamin B_1-free diet; *e.g.* Coward, Burn, Ling and Morgan (1933) found that 8% of autoclaved yeast in a vitamin B_1-free diet did not supply enough vitamin B_2 to obtain the full effect of doses of the International Standard for vitamin B_1, although they obtained graded responses to graded doses of the Standard. When, however, 20% of the autoclaved yeast was used, ample amounts of vitamin B_2 were supplied. This dried yeast was autoclaved in layers about 0·1" thick at 120° C. for 6 hours. A direct comparison between dried yeast autoclaved thus and an untreated portion of the same sample showed that this process of autoclaving had destroyed about 50% of some factor other than vitamin B_1 in the dried yeast. Therefore, since 8% of dried yeast provides abundance of all the B vitamins, 20% of yeast, autoclaved as described, will provide abundance of the B vitamins other than B_1 with a fair margin of safety.

4. Criteria for the Measurement of the Response of Animals to Doses of Vitamin B_1

Perhaps the most generally used criterion for the measurement of vitamin B_1 is the cure of " retracted neck " in pigeons.

When these birds are fed on a diet of polished rice and water, and kept in cages with screens of wire mesh raised about 2" from the floor to prevent access to fæces, 40–50% will develop " retracted neck " in 3 or 4 weeks. The head suddenly jerks backwards so that the top of the skull rests in the feathers of the bird's back. It may recover its normal position again for a time, but when the condition becomes worse the neck remains retracted. The head can be moved about a little from side to side and can be held in its natural position by an operator without apparently giving the bird any pain. If no dose of vitamin B_1 is given the bird will die within about 48 hours. If, however, a dose of, say, 0·5g. dried yeast is given in a little water by pipette into the bird's throat it recovers in about 12 hours. If after that it is given no more vitamin B_1, it will again develop a retracted neck in a few days, or it may suddenly die.

The birds ought to be looked at three times a day. When one is found with a retracted neck it may be given the desired dose, and put in a smaller cage alone and still in the open air. It should be given food (the vitamin B_1 deficient diet) and water. If it is cured within 24 hours that may be considered a 1-day cure. If it remains cured for another 24 hours, that may be considered a 2-days cure, and so on. Parts of days must be ignored unless the birds are watched all night both for development of retracted neck and for cure. The cure is quick as a rule but not sudden. The neck may regain its normal position for a short time and then revert to the abnormal position, but if the dose is really curing the bird the head will be normal in 24 hours. It really is useless to try to refine the calculation more than this.

Some workers (Kinnersley, Peters and Reader, 1928) find it useful to give a pigeon a small dose of glucose in water and then wait a few hours to see whether that will bring about a " spontaneous cure " of the bird before giving it a dose of test substance or of Standard. A few birds may respond to this treatment and develop the true polyneuritis a few days later.

When rats are fed on a diet which contains all substances known to be necessary for growth except vitamin B_1 they will cease to put on weight in 1–2 weeks and if still no vitamin B_1 is given to them they will lose weight and die in 2–3 weeks. If, on the other hand, they are given a daily dose of a substance

containing vitamin B_1 they will increase in weight at a rate proportional to the log. of the dose of vitamin B_1 given.

M. I. Smith (1930) has produced polyneuritis in rats by feeding them on a diet "so constituted as to include the thermostable factor but lacking in the antineuritic vitamin." He says this condition results almost invariably after 6–8 weeks of feeding on the deficient diet. Furthermore, the administration of a yeast concentrate containing the antineuritic vitamin brings about prompt and complete recovery from the paralytic symptoms, the duration of the remission being from 3–15 or more days according to the size of the dose administered. The paralysis will recur again if no further vitamin B_1 is given but may again be alleviated. The paralytic condition is characterised by lameness of the hind- and fore-limbs, incoordination, spastic gait, cart-wheel and rolling movements. Intravenous injection of concentrates into a tail vein is recommended as being more certain than oral administration.

Drury, Harris and Maudsley (1930) and Birch and Harris (1934) have used the cure of bradycardia for measuring the vitamin B_1 content of a substance. When young rats are given a vitamin B_1 deficient diet their heart-rate falls. In 3 weeks it has generally fallen from about 500 or 550 per minute to 350 per minute. When a single dose of vitamin B_1 is given the heart-rate is increased, reaching the maximum obtainable on the given dose in about 24 hours, after which, if no further vitamin B_1 is given, the rate again declines. The rate is determined by means of an electrocardiograph.

A. **Cure of retracted neck in pigeons.**

This is the simplest biological test known for any vitamin. The preparation of the birds is simple, one dose only is administered to each bird and the result is available the next morning if a cure is the only figure required, or in a few days if the duration of cure is required.

(*a*) *Preparatory period.*—Pigeons suitably housed and given a diet of polished rice and water will usually develop retracted neck in 20–30 days if they do so at all. About 50% of pigeons never develop retracted neck and are therefore wasted in a vitamin B_1 test.

(*b*) *Dosing of the pigeons.*—The Standard and most test substances can be given to the pigeons very easily. The dose

of Standard is weighed on a watch-glass and about 1ml. water added. This is drawn up into a clean dry pipette with a short length (about 2″) of fine rubber tubing on the end and allowed to run out into the throat of the pigeon whose neck is held extended with the left hand. A little more water is poured on to the watch-glass and this is taken up in the pipette and given to the pigeon in the same way. If necessary, a second washing may be given.

Bulky substances of low vitamin B_1 content may be given by forcible feeding down a glass tube passed into the œsophagus of the bird. The food is finely ground and rammed down the tube by a narrow glass rod.

Occasionally it is found that a pigeon cannot tolerate a substance and will vomit it. This pigeon should be rejected. If all the pigeons behave in the same way to any particular substance, the pigeon test will have to be abandoned and a rat test tried.

(*c*) *Working out the result.*—(i) Suppose the following figures had been obtained in a pigeon test as described above :

Dose.	Percentage birds cured.	Average duration of cure (days).
0·02g. International Standard	30	2·1
0·04g. International Standard	60	6·5
0·1g. substance X	3	0·5
0·2g. substance X	10	1·3
0·4g. substance X	35	2·0

The substance X has proved to be less rich in vitamin B_1 than was anticipated, but 35% of birds cured corresponds well enough to 30% birds cured to say that the substance X contains about 5 International units of vitamin B_1 per gram (0·4g. substance X being regarded as equal to 0·02g. International Standard which is equivalent to 2 units). A similar result is obtained by comparing the average duration of cure in the different groups.

(ii) Suppose a curve of response has been constructed (*e.g.* the curve in Fig. 43 constructed by Coward, Burn, Ling and Morgan, 1933), and in the same laboratory in a determination of the vitamin B_1 content of a certain substance Y, 43% of the

birds given 0·03g. International Standard were cured, 69% of the birds given 1·0g. substance Y were cured.

On the curve of response relating the percentage of birds cured to dose of vitamin B_1 given, the abscissa corresponding to 43% birds cured was 0·023; the abscissa corresponding to 69% birds cured was 0·50.

Thus the ratio $\frac{\text{potency of 1·0g. substance Y}}{\text{potency of 0·03g. International Standard}}$ is not $\frac{69}{43}=1{\cdot}6$ but $\frac{0{\cdot}050}{0{\cdot}023}=2{\cdot}2$, and the potency of substance Y is therefore about 6·5 International units of vitamin B_1 per gram.

Similarly, the average duration of cure of the birds given 0·03g. International Standard was 3·5 days and that of the birds given 1·0g. substance Y was 5·0 days. Therefore the ratio of the potencies of the doses was found from the curve relating duration of cure to dose of vitamin B_1 given (Fig. 11) to be not $\frac{5{\cdot}0}{3{\cdot}5}$ $(=1{\cdot}4)$ but $\frac{0{\cdot}074}{0{\cdot}039}$ $(=1{\cdot}9)$. The potency of the substance Y may therefore be said from either test to be about 6 International units of vitamin B_1 per gram.

(iii) *Peters' "day-dose" calculation.*—If Peters gives a dose of xg. of a substance to a pigeon and finds that it is cured for y days, the potency of the substance is said to be y/x day-doses per gram. Hence if the figures given in the last example were used according to Peters' method, the International Standard would contain $\frac{3{\cdot}5}{0{\cdot}03}=117$ day-doses per gram and the substance Y would contain $\frac{5{\cdot}0}{1{\cdot}0}=5$ day-doses per gram. Thus the substance Y would have $\frac{5}{117}$ of the vitamin B_1 potency of the International Standard. As this contains 100 units per gram, the substance Y would be said to contain $\frac{5}{117}$ of 100 units $=4{\cdot}3$ units per gram. This result is less than the one calculated by the use of a curve of response which is accounted for by the fact that the day-dose method of calculating the result assumes, by the use of simple proportion sums, that the curve of response

relating duration of cure to dose of vitamin B_1 given is a straight line. Now Coward's curve of response may be regarded as practically a straight line throughout a short part of its length. If both results fell on this short part, the day-dose method of calculating the result would give the same answer as that obtained by the use of the curve, but if one or both results fell on parts of the curve outside this straight line part, then a different answer would be obtained. Obviously the only safe method of calculation, unless the two results are nearly equal, is to use a curve of response.

B. **Increase in weight in rats.**

(*a*) *Preparatory period.*—Rats require a shorter period in which to exhaust their reserves of vitamin B_1 than the period they require to exhaust their reserves of vitamin A or D. They generally become steady in weight in 10–14 days of feeding on the vitamin B_1-free diet. They should be weighed twice a week during this time and put on experiment within a day or two of the first fall in weight, for on a shortage of vitamin B_1 rats quickly lose weight and die.

(*b*) *The test period.*—There is no need to carry on a rat test for vitamin B_1 longer than 3 weeks. Indeed, Coward (1936) has shown that a test carried on for 2 weeks gives a result almost as accurate as one carried on for 3 weeks. The accuracy obtainable in a vitamin B_1 test is certainly greater than that obtainable in a vitamin A test in the same length of time.

During the test period doses of the test substance and of Standard may be given daily or half-weekly. In three different experiments, one with dried yeast and two with different adsorbates of vitamin B_1 similar to the International Standard, Lindholm, Laursen and Morgan (unpublished results obtained in the writer's laboratory) have found these two ways of giving the doses to produce equal responses.

The giving of daily doses of a potent substance such as the International Standard can be simplified by making a 10% dilution of it with finely powdered, dry, dextrinised rice starch. These in the proportion 1 : 9 should be very intimately mixed by grinding together in a mortar. Then a dose of the mixture ten times the weight of the dose of standard required can be quickly weighed on a watch-glass and transferred to a small dish in which it may be given to the rat. The powder may be

moistened with water immediately before it is put in the rat's cage. The rat always takes it eagerly; thus there is no danger of deterioration through the vitamin B_1 remaining long in contact with water, and there is no loss through the scattering of the powder.

Other substances less potent than the International Standard may be weighed directly, transferred to a small dish and moistened to prevent scattering. Dried yeast, wheat embryo, etc., are conveniently given to the rats in this way.

Other substances of still lower potency may be mixed with the diet in the required percentage assuming that a rat of 50–60g. weight eats about 5g. food (dry weight) per day. Records of food consumed should then be made in order to determine the exact weight of test substance eaten during the full period of the test. Food-pots with turned-in rims should be used to prevent scattering. Ten grams of food may be given on the first day of the test, the part not eaten by the next morning weighed and made up to 10g. again or more if necessary. There should always be a surplus of at least 2g. in the pot in the morning.

The rats may be weighed only once a week during the test period.

(*c*) *Working out the result.*—(i) When two or three doses of the test substance have been given to different groups of rats, and two doses of the International Standard to two other groups, the average increases in weight of the rats of the different groups are calculated and compared. The following figures were obtained in a test carried out as described above:

Substance.	Dose, g.	No. of rats in test.	Mean increase in weight in 3 weeks, g.
X	0·5	5	15·4
X	1·0	6	29·0
International Standard	0·01	5	11·6
International Standard	0·02	6	23·2

It is evident that 0·5g. substance X contains a little more vitamin B_1 than 0·01g. of the International Standard; that is, 0·5g. X contains a little more than 1 International unit.

Similarly, 1·0g. substance X contains a little more vitamin B_1 than 0·02g. International Standard; that is, 1·0g. X contains

a little more than 2 International units. But 0·5g. X is far from being equal to 0·02g. of the Standard. Thus the nearest approximation to the true vitamin B_1 potency of substance X that can be made from these figures is probably about 2·3 International units per gram.

(ii) These figures may, however, be worked out in a different way. They were obtained in Coward's laboratory with the same basal diet as was used in the construction of a curve of response to doses of vitamin B_1 in that laboratory. For the curve different groups of rats had been given graded doses of the International Standard, 0·005, 0·01, 0·02, 0·04 and 0·1g. respectively. Equal numbers of male and female rats had been used in each group. The rats were given the vitamin B_1-free diet until they ceased to put on weight. Then they were distributed as evenly as possible into the five groups, and given the appropriate dose of Standard daily. The mean increase in weight of each group of rats during 3 weeks' dosing with the Standard was calculated and the averages so obtained were plotted against the daily dose of Standard given. Thus a curve relating mean increase in weight of rats in 3 weeks and dose of vitamin B_1 given, was obtained (Fig. 12). This curve may be used for working out the last determination as follows :

Substance.	Dose, g.	No. of rats given dose.	Mean increase in weight of rats in 3 weeks, g.	Abscissa corresponding to mean increase in weight.
X	0·5	5	15·4	0·018
X	1·0	6	29·0	0·027
International Standard	0·01	5	11·6	0·016
International Standard	0·02	6	23·2	0·023

From this it may be seen that the ratio $\frac{15{\cdot}4}{11{\cdot}6}$ for increase in weight corresponds to the ratio $\frac{0{\cdot}018}{0{\cdot}016}$ for dose of vitamin B_1 given. Thus :

$$\frac{\text{0·5g. substance X}}{\text{0·01g. International Standard (1 unit)}} = \frac{0{\cdot}018}{0{\cdot}016} = 1{\cdot}125$$

Therefore 0·5g. X contains $1{\cdot}125 \times 1 = 1{\cdot}125$ International units, and 1·0g. X contains 2·25 International units. Similarly

the ratio $\frac{29{\cdot}0}{23{\cdot}2}$ for increase in weight corresponds to the ratio $\frac{0{\cdot}027}{0{\cdot}023}$ for dose of vitamin B_1 given. Thus:

$$\frac{1{\cdot}0\text{g. substance X}}{0{\cdot}02\text{g. International Standard (2 units)}} = \frac{0{\cdot}027}{0{\cdot}023} = 1{\cdot}17$$

Therefore 1·0g. X contains $1{\cdot}17 \times 2 = 2{\cdot}34$ International units. The average of the two results is about 2·3 International units per gram. Thus the result obtained by the more accurate method of calculating it confirms the result obtained by the simpler method.

The advantage of having a curve of response for the interpretation of results lies in the fact that with it fewer animals can be used, one group only being given a dose of the test substance and one group a dose of the Standard. The comparison is made through the abscissæ corresponding to the mean increases in weight of the two groups, just as two comparisons were made with two pairs of groups in the example worked out above.

(iii) Even if no curve of response had been available for calculating the potency of substance X from these results, the potency could be calculated more accurately than in the first very simple method thus:

It may be assumed that the curve of response of rats to doses of vitamin B_1 is logarithmic, *i.e.* if the increases in weight are plotted against the logarithms of the doses given and the points joined, a straight line will be formed. Therefore the mean increases in weight from 0·01g. and 0·02g. Standard were plotted against the logarithms of the doses given. A straight line was drawn between these points and produced upwards a little (Fig. 13). The abscissæ corresponding to the mean increases in weight from the doses of substance X were determined and the calculation completed as in Table VI.

TABLE VI

Dose.	Mean increase in weight in 3 weeks, g.	Abscissa corresponding to mean increase in weight.	Antilog. of abscissa.	Potency of X according to each result.
0·5g. X	15·4	$\bar{2}{\cdot}098$	0·0125	2·5 units per gram.
1·0g. X	29·0	$\bar{2}{\cdot}448$	0·0280	2·8 units per gram.

According to this calculation the potency of substance X is 2·65 (average of 2·5 and 2·8) International units per gram. There is no need to decide which is the more accurate method of calculating the result, for the two answers are near enough to be regarded as the same.

The potency of substance X may be determined from these figures without actually drawing any curve of response. The two points obtained from the doses of Standard have the values

(*a*) $x_1, y_1 = 0{\cdot}01, 11{\cdot}6.$

(*b*) $x_2, y_2 = 0{\cdot}02, 23{\cdot}2.$

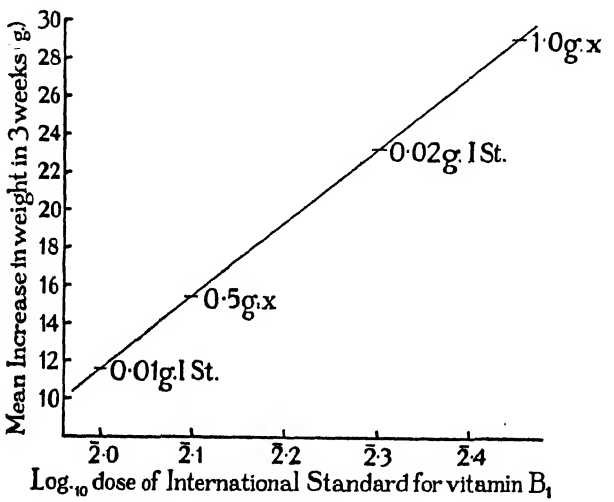

FIG. 13.—Curve of response relating mean increase in weight of rats in 3 weeks to doses of 0·01g. and 0·02g., respectively, of the International Standard for use in the calculation of the potency of substance X tested in doses of 0·5g. and 1·0g. at the same time.

The slope of the curve of response is obtained by substituting values of y_1 and y_2 and the logs. of the values of x_1 and x_2 in the formula $\frac{y_2-y_1}{x_2-x_1}$ which gives $\frac{23{\cdot}2-11{\cdot}6}{2{\cdot}3010-2{\cdot}0} = \frac{11{\cdot}6}{0{\cdot}3010} = 38{\cdot}54.$

The calculation of the slope need not, however, be carried quite so far as this. The fraction gives the information that a difference of 11·6 in the increase in weight corresponds to a difference of 0·3010 in the log. of the dose of Standard given. Therefore, a proportion sum gives the information that a difference of 15·4—11·6g., *i.e.* 3·8g. (the difference between

the increases in weight brought about by 0·5g. substance X and by 0·01g. Standard respectively) corresponds to a difference of $\frac{0{\cdot}3010 \times 3{\cdot}8}{11{\cdot}6} = 0{\cdot}0986$ in the logs. of the doses given. This is the log. of 1·255 which means that the vitamin B_1 content of 0·5g. X is 1·255 times the vitamin B_1 content of 0·01g. Standard. Therefore 0·5g. X contains 1·255 units of vitamin B_1, and therefore X contains 2·5 International units per gram.

Similarly a difference of 29·0–23·2g., *i.e.* 5·8g. (the difference between the increase in weight brought about by 1·0g. X and by 0·02g. Standard respectively) corresponds to a difference of $\frac{0{\cdot}3010 \times 5{\cdot}8}{11{\cdot}6} = 0{\cdot}1505$ which is the log. of 1·415. Thus the vitamin B_1 content of 1·0g. X is 1·415 times the vitamin B_1 content of 0·02g. Standard. Therefore 1·0g. X contains 2·8 International units of vitamin B_1.

The average of these two results (2·5 and 2·8) is 2·65 International units per gram, which is the result obtained by the previous method of calculation.

C. **Cure of convulsions in rats.**

The writer has had no success in attempts to produce convulsions in rats. Therefore the following details of this method are based on work by Birch and Harris (1934) :

(*a*) *Preparatory period.*—If rats of 70–80g. weight are given no vitamin B_1 in their basal diet they will die without developing polyneuritic convulsions, but if given traces of vitamin B_1 (enough just to keep them alive) they will develop this symptom. Thus a very small dose of vitamin B_1 should be given to each rat during the preparatory period (Sherman and Sandells, 1931, Sebrell and Elvove, 1931). In 6–8 weeks the polyneuritic condition generally becomes evident (Birch and Harris, 1934).

(*b*) *Dosing of the rats.*—Doses of test substance or of Standard may be given by mouth. If a comparison of concentrates is to be made, the doses may be injected intraperitoneally. As the rats become ready for the test they are distributed into, say, four groups, two groups for doses of the Standard in the ratio 2 : 1 and two groups for doses of the test substance. The latter doses should be of a very wide range if no information is available of the probable potency of the substance, but when

that information has been obtained, a narrower range of doses is tested against a fresh test of two doses of the Standard.

(*c*) *Working out the result.*—The average number of days that the rats remain cured after receiving a dose is calculated for each group. The most satisfactory determination is made when the average result from a dose of the Standard is equal to the average result from a dose of the substance under test. If no two average results correspond, then the calculation is made as in the increase in weight method, viz. by a predetermined curve of response or by constructing a curve from the pair of results from the two doses of Standard. As, however, Birch and Harris showed that a straight line relationship exists between dose and duration of cure of convulsions, simple proportion sums will give the answer as easily as a curve of response.

D. **Cure of bradycardia in rats.**

This method is based on the details given in the papers by Drury, Harris and Maudsley (1930), and Birch and Harris (1934).

(*a*) *Preparatory period.*—Young rats of about 40g. weight are given a vitamin B_1-free diet. After about 3 weeks electrocardiograms are taken. The unanæsthetised rat is stretched on a board, its head held firmly in a clamp, and its legs held out by slip nooses wound round cleats on the sides of the boards. One of the electrodes, a small needle, is placed under the skin of the right foreleg, the other at the lower end of the thorax. The needles are connected to a Matthew's portable electrocardiograph on which permanent photographic records are then taken. When the heart-rate is down to about 350 per minute the rat is ready for the test.

(*b*) *Dosing of the rats.*—As the rats become ready for the test, two groups are given doses of the Standard in the ratio 2 : 1 and two are given doses of the test substance. If some information of the probable potency of the substance to be tested is available, then the doses may be in the ratio 2 : 1. If no such information is available, then a preliminary test must be made with doses in a wider range, say, of 3 : 1, followed by another test with doses in the ratio 2 : 1 more nearly equivalent to the doses of Standard, which must of course be tested again simultaneously with the two doses of test substance.

There is not enough material available for an estimation of the number of rats that should constitute each group in this test, but it is safe to recommend at least 5 rats for a group when two groups are used for the Standard and two groups for the test substance.

A single dose only of the Standard (or substance tested) is given to each rat. Its diet is the same as before. Its electrocardiogram is taken again 24 hours after dosing. If the rate has not increased a larger dose is tried. With a large enough dose, the rate rises to normal in 24 hours and then gradually slackens again. The increase in rate in 24 hours, and the time taken to slow down to the rate when the dose was given are both measures of the potency of the dose given. Each is roughly proportional to the dose of vitamin B_1 given. The relationship between dose and effect appears to be a straight line. Cures of 4 days' duration are a suitable basis of comparison at which to aim.

(*c*) *Working out the result.*—Since the relationship between effect and dose of vitamin B_1 given is a straight line, the potency of the substance tested may be worked out by simple proportion sums from the average duration of the cures from the different doses of Standard and of substance tested.

E. Comparison of the four methods.

The cure of retracted neck in pigeons, the cure of convulsions in rats and the cure of bradycardia in rats all appear to be specific for vitamin B_1 which gives these methods a very great advantage over the "increase in weight" method with rats.

The retracted neck method requires 3–4 weeks to prepare the birds but only one day after the giving of each dose to get a result, if the result is calculated from the percentage of birds cured. If the result is calculated from the duration of cure a few more days are necessary. If enough pigeons were kept in continuous preparation for the test the length of time required for a test would be decided by the longest period of cure of any bird. It is therefore a very quick test. It is also a very simple test, for the birds require very little attention during their preparatory period, beyond the giving of clean water and fresh rice each day and the cleaning of their cages twice a week. Also, only one dose is given to each bird.

The cure of convulsions in rats requires a longer time to

prepare them for the test but, provided a large enough supply of rats is kept in preparation a test can be completed in as short a time as by the " cure of retracted neck in pigeons " method. The cure of bradycardia in rats requires about 3 weeks of preparation, but again, only one dose is given to each animal, unless the dose is seen next day to be ineffective, when a larger dose is given. The time taken to complete the test is about the same as that required by the " cure of convulsions in rats " method. The cost of the electrocardiograph would, however, be prohibitive for many laboratories. The " increase in weight in rats " method requires about 2 weeks for the preparation of the rats, and then a daily or half-weekly dose of the substance under test or of the Standard, for 2 weeks at least. Hence a longer time is required for obtaining a result than is required by either of the other methods and more labour is required for the giving of doses. It is not specific for vitamin B_1 since so many other factors are required for an increase in weight. It is, however, very much more accurate than the pigeon method and probably also than the other two methods, but that consideration should not make one lose sight of the point that the other three criteria appear to be specific for vitamin B_1 while the " increase in weight " is not.

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CHAPTER V

THE DETERMINATION OF VITAMIN C

1. The International Standard of Reference and the Unit of Vitamin C Activity.
   - A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin C is made.
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THE existence of vitamin C as " something in lemon juice and fresh vegetables that prevents scurvy " has been recognised longer than any other vitamin.

Rats do not need to be given vitamin C in their diet. It is, however, found in their livers, even after a long period on a diet deficient in this factor. A recent investigation by Hopkins (1934 and 1935) showed that vitamin C is found in the walls of the small intestine where it is probably synthesised. Thus rats cannot be used for the determination of vitamin C. Guinea-pigs, however, cannot synthesise this factor. When fed on a diet deficient in vitamin C, they begin to lose weight after about 18 days and generally die within 28 days, with macroscopic and microscopic symptoms of scurvy. If, however, they are given vitamin C (ascorbic acid) in sufficient quantities in addition to this diet, they will grow and will not develop scurvy. Hence guinea-pigs are useful animals for the biological determination of vitamin C.

Since the discovery by Svirbely and Szent-Györgyi (1932) of the antiscorbutic properties of a crystalline substance which has since been named ascorbic acid, much work has been done on the determination of the ascorbic acid content of animal and vegetable tissues by making use of its reducing action on 2, 6-dichlorphenol-indo-phenol (Tillmans, Hirsch and Jackisch, 1932). The reaction is, however, not specific for vitamin C; it is known to be given by certain other substances such as glutathione and adrenaline. For animal tissues Hopkins, Slater and Millikan (1935) have worked out a modification of the method which eliminates the influence of these two substances.

Kon and Watson (1936) showed that when milk was exposed to light for a short time an oxidised form of ascorbic acid, dehydroascorbic acid, was formed which was biologically active but without action on 2, 6-dichlorphenol-indo-phenol. They proposed reducing this substance with sulphuretted hydrogen before titration. Mack and Tressler (1937) showed that the formation of the oxidised form in vegetable extracts could be prevented by the addition of a strongly ionised acid which destroyed the oxidising enzyme. The reduction with H_2S was thereby eliminated. The concentration of a pure solution of ascorbic acid can be determined by the intensity of its absorption at 245mμ, but in impure solutions the intensity may be modified by other substances. Until the ascorbic acid content of all food substances can be determined by chemical or physical methods the need for a biological method of determination remains.

1. The International Standard of Reference and the Unit of Vitamin C Activity

The Standard of reference recommended for adoption by the Permanent Commission on Biological Standardisation of the League of Nations in 1931 was the freshly expressed juice of the lemon, *Citrus limonum*. The unit of vitamin C activity was 0·1ml. of the fresh juice. The view was accepted that this was less variable in potency than any concentrate available. The opinion as to its constancy of vitamin C content was based on the evidence that a daily dose of 1·0–1·5ml. of fresh juice was always found to be sufficient to protect guinea-pigs from scurvy for 60 or even 90 days. It was recognised at the time

that this was a bad method of judging the constancy of the potency of the juice, for any "complete" animal reaction, other than the death of the animal, is very difficult to judge. Moreover, if a dose of 1·5ml. juice had brought about complete protection, 1·25ml. or 1·0ml. might also have brought it about. It is therefore easily understandable that with "complete protection" as the criterion, early workers on vitamin C were led to believe that lemon juice had a constant vitamin C content. Since the discovery that vitamin C is a substance (ascorbic acid) which can be measured chemically in lemon juice with accuracy, it has been shown that the potency of lemon juice varies greatly. Bacharach, Cook and Smith (1934) in an examination of 15 lemons found extreme values of 0·47 and 0·73mg. per ml., with a mean of 0·64mg. per ml. of the juice.

The Standard of reference recommended for adoption by the Permanent Commission on Biological Standardisation of the League of Nations in 1934 was *l*-ascorbic acid of which Professor Szent-Györgyi was asked to prepare a sample of 500g. Professor W. N. Haworth of the University of Birmingham was invited to co-operate in controlling the purity of the Standard material. No special precautions are required for preserving the activity of the Standard. It may be kept at room temperature in a sealed tube or in an evacuated desiccator. The part needed for dosing must be removed as required each day; no solution must be kept from day to day (see below).

The unit of vitamin C activity recommended for adoption is the vitamin C activity of 0·05mg. *l*-ascorbic acid. This is approximately the average amount of *l*-ascorbic acid in 0·1ml. of fresh lemon juice. Thus the biological value of the unit of vitamin C activity remained unchanged through the changing of the Standard.

A. **The need for a simultaneous test on the Standard of reference whenever a determination of vitamin C is made.**

There is only one reference in the literature to the variation in response of guinea-pigs at different times to the same dose of vitamin C. Key and Morgan (1933), using the method of determination developed by Key and Elphick (1931), and testing a series of five doses ranging from 0·15 to 2·0mg. of ascorbic

acid, at three different times, found that the slopes of the curves of response to these doses was the same as the slope of the curve previously obtained by testing a series of doses of orange juice, but that the positions of the curves differed slightly. This was a good indication of a variation in sensitivity of the guinea-pigs used. Further work (unpublished results) from the writer's laboratory has confirmed it (Fig. 14).

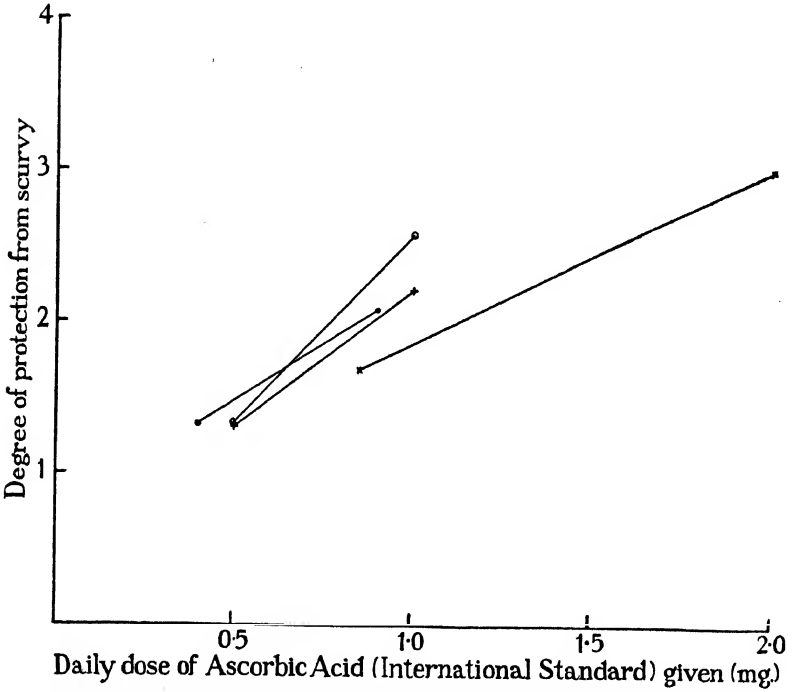

FIG. 14.—Curves of response to doses of vitamin C obtained at different times.

This result, together with the fact that all investigated animal reactions are known to show variations in intensity from time to time, make it quite evident that whenever a determination of vitamin C is to be made, a simultaneous test of the Standard must be made on similar animals.

B. **The solution of the International Standard of reference for dosing.**

It is of the utmost importance that the Standard of reference (ascorbic acid) should be dissolved in freshly boiled and cooled,

glass-distilled water immediately before it is given to the guinea-pigs each day. A solution of ascorbic acid in water from a copper still will lose much of its vitamin C activity within an hour.

C. **The general arrangement of the test for a determination of the vitamin C potency of a substance in terms of the International Standard.**

Whatever the criterion chosen for the measurement of the vitamin C potency of a substance, the arrangement of the experiment is the same. Prophylactic (preventive) experiments are usually made for vitamin C though sometimes an economy of labour is effected by giving all the guinea-pigs no supplement of Standard or test substance for 10–14 days at the beginning of the experiment.

When it is expected that only an occasional determination of vitamin C is to be made, it is probably not worth while to construct a curve of response to different doses. It would certainly be worth while to construct a curve of response if half a dozen determinations had to be made.

Without a curve of response, the test should be arranged as in similar determinations of other vitamins. Five groups of 5 guinea-pigs each should be used. Two should be given doses of the Standard in the ratio 2 : 1 and three should be given doses of the test substance in the ratio 9 : 3 : 1 if no information is available of the probable potency of the substance. When such information has been obtained, then two doses only, in the ratio 2 : 1 need be tested. Every member of any one group is given the same dose. None of the doses of Standard chosen should be great enough to afford complete protection. The object is to find one dose of the test substance giving the same results as one dose of the Standard, or possibly two doses of the test substance giving the same results as two doses of the Standard. If the scurvy developed from all three doses of test substance was more severe than that developed from the lower dose of the Standard or less severe than that developed from the higher dose, then the test would have to be repeated with a different range of doses of test substance, and possibly with a different range of doses of the Standard.

To construct a curve of response, five groups of guinea-pigs of 10 animals in each group are needed. The groups are

given 0·125, 0·25, 0·5, 1·0 and 2·0mg. respectively of ascorbic acid, every animal of any one group being given the same dose. Apparently there is no need to distinguish between male and female guinea-pigs in the determination of vitamin C by the " tooth " method. The results in each group are averaged and the means are plotted against the doses of vitamin C given. When this has been done, tests can be carried out on two groups of guinea-pigs only, one being given a moderate dose of the Standard and the other a dose of test substance which is likely to contain about as much vitamin C as the dose of Standard chosen. The ratio of the potencies of these doses is, then, not the ratio of the averages of the response in the two groups, but the ratio of the abscissæ of the curve of response corresponding to these average results.

2. The Preparation of Guinea-pigs for a Vitamin C Determination

A. Animals suitable for the test.

Young guinea-pigs weighing 200–250g. are used for vitamin C determinations whatever may be the criterion chosen for measuring the effect of the doses of test substance and Standard. It is not necessary that they should be bred in the laboratory in which they are to be used, but it is very essential that they should be obtained from a healthy stock. It is not necessary apparently to have the same proportion of bucks to does in all groups of a test in which the " tooth " method is used for diagnosis, but it is important to have the same proportion of bucks to does in all groups when the " increase in weight " method is used.

B. Housing of the guinea-pigs.

The laboratory for animals used in a vitamin C test should be light, airy and warm, for guniea-pigs easily develop pneumonia. Their susceptibility to the disease is a strong argument against long-time methods of testing for vitamin C, for the longer the test period the greater is the chance of intercurrent diseases affecting the results. For long-time tests Bracewell, Hoyle and Zilva (1930) have found it useful to inoculate all animals with a vaccine consisting of equal numbers of *B. enteritidis*, Gaertner and *B. ærtrycke* before the experiment to eliminate intercurrent diseases of intestinal origin.

C. **Diets suitable for vitamin C tests.**

(1) A simple diet which has proved satisfactory for vitamin C work in many laboratories has the following composition:

Bran	45%
Crushed oats	25%
Dried skimmed milk	30%

In addition each guinea-pig is given about 1ml. cod liver oil twice weekly by pipette into the back of its mouth. Tap water is supplied in an open feeding pot which cannot easily be upset. The food itself is supplied *ad lib.* in a feeding pot with a turned-up top to prevent spilling. It is not a homogeneous mixture but in practice that seems to be immaterial.

(2) The diet used by Bracewell, Hoyle and Zilva (1930) in their study of the vitamin C content of apples consisted of:

Bran	6 parts by volume.
Barley-meal	2 „ „
Middlings	3 „ „
Fish-meal	1 part by volume.
Crushed oats	4 parts by volume.

This was given *ad lib.* in addition to 40–60 ml. of autoclaved milk made up from a dried full-cream powder. No cod liver oil was given with the diet, the vitamin A needed by the pigs being supplied by the fish-meal and milk.

3. Criteria for the Measurement of the Response of Guinea-pigs to Doses of Vitamin C

(*a*) *Onset of scurvy.*—The symptoms of scurvy noticeable in the living guinea-pig are stiffness of the joints, particularly of the hind legs, a tendency to sit crouched up and, later, the "face-ache" position of the head, cessation of growth and loss of weight. Death of the animal will follow within 28 days of the withholding of vitamin C from the diet. The symptoms are more easily recognised by the trained worker than described. If guinea-pigs are killed when they first appear to be developing scurvy, slight hæmorrhages are found at the joints, particularly at the knees, the ribs are beaded and the gums are swollen and may have hæmorrhages also. These macroscopic lesions may easily be classified as slight, severe, very severe, etc., and a rough comparison can be made between the groups of animals that have received different doses.

(*b*) *Histological changes in the teeth.*—Zilva and Wells (1919) first showed that guinea-pigs fed on a scorbutic diet developed

changes in the structure of the teeth. Höjer (1924, 1926) examined these changes and claimed that they could be measured so that a quantitative determination of vitamin C could be based on them. Goettsch (1928) thought that Höjer had claimed too great a degree of accuracy for his method, and Key and Elphick (1931) drew up a scale of disordered teeth (Figs. 17–21) (based partly on Goettsch's work) and a curve of response (Fig. 15) to graded doses of vitamin C, by means of which more accurate comparisons could be made than had hitherto been possible. The accuracy obtainable by

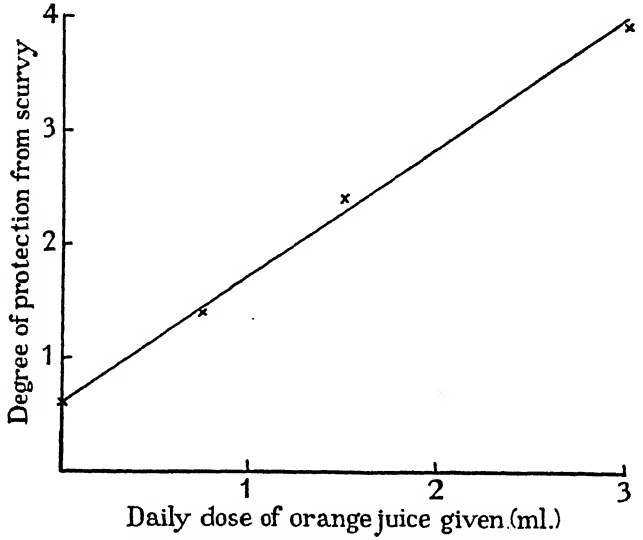

FIG. 15.—Curve of response relating the amount of protection from scurvy and the amount of vitamin C (as orange juice) given. (Constructed by Key and Elphick.)

the method was examined by Coward (1934), and found to be very similar to that obtainable in other vitamin tests.

(*c*) *Increase in weight of the guinea-pig.*—Zilva has used the increase in weight in 60–90 days of guinea-pigs given a scorbutic diet plus the substance containing the antiscorbutic factor as a means of comparing the vitamin C potency of different substances. He states that guinea-pigs fed on his scorbutic diet alone will die within 28 days, and that the increase in weight of other guinea-pigs given the vitamin is proportional to the dose of vitamin given. In comparing the vitamin C

potency of different kinds of apples, and of apples of the same kind grown under different conditions, or stored under different conditions, he has equated the doses that gave approximately the same rates of growth in his animals.

Coward and Kassner (1936) modified this method in two respects: (*a*) a simultaneous test on the International Standard of reference was made, (*b*) the period of the test was shortened to 6 weeks.

A. **Key and Elphick's modification of Höjer's "tooth" method.**

Höjer claimed that he was able to distinguish between doses of vitamin C differing by as little as 10%, by examination of the teeth of the guinea-pigs used.

When Goettsch (1928) tried this method, using a 2 weeks' test period, she found that two doses differing by as much as 300% might bring about equal responses in 2 animals. This is no greater than the variation found in most other animal reactions but it means that very large numbers of animals would have to be used and the results averaged to distinguish between doses differing only by 10%.

Key and Elphick (1931) showed that a graded response to graded doses of vitamin C could be obtained by using groups of about 5 animals for each dose tested. The results obtained from the different pigs of a group were averaged by comparing the tooth section of each pig with an arbitrary scale which they had drawn up depicting teeth in different stages of the disorder brought about by a lack of vitamin C. (Figs. 17–21). The use of this method then follows the general plan of a vitamin determination.

(*a*) *Dosing the guinea-pig.*—Whenever possible, the dose of substance to be tested should be given directly into the guinea-pig's mouth. When the substance is a fruit juice this can easily be done from a pipette and generally fruit juices are so potent that the dose needed for the test is small and can be given quickly. The guinea-pig is allowed to sit comfortably on a table, its head firmly held in one position and the end of the pipette placed far back in its mouth. No attempt should be made to make the guinea-pig swallow his dose more quickly than he wishes to, or part of the dose may be lost. If the volume of liquid to be given is large it may be given in two or three parts at intervals of a few hours. This plan is useful

when milk has to be tested, for the vitamin C value of milk is low.

The International Standard is easily handled. It is soluble in water and the required weight can be given to a guinea-pig conveniently in a volume of 2ml. water. The precaution mentioned in the description of the Standard may be repeated here. The solution should be made up immediately before being given to the pigs and only freshly prepared, glass-distilled water should be used.

Doses should be given daily for 14 days, with double doses on Saturday and none on Sunday.

(*b*) *Preparation of the teeth for examination.*—At the end of the 14 days' period the guinea-pigs are killed and examined for any macroscopic symptoms of scurvy. It is unusual to

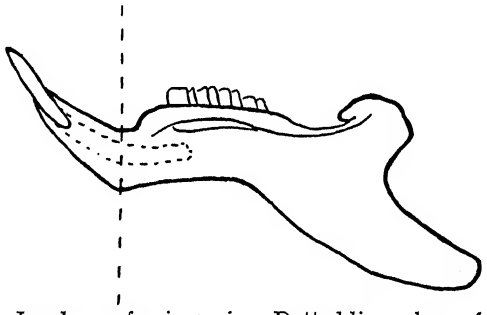

FIG. 16.—Jaw-bone of guinea-pig. Dotted line, plane of section.

find any symptoms as they seldom develop in so short a time as 14 days of feeding without vitamin C. The absence of these lesions is a good indication that the animals were not in a scorbutic state at the beginning of the experiment.

The lower jaw-bone is removed and freed as completely as possible from adhering muscle and skin. It may be divided into two by cutting between the incisors. The parts are placed in decalcifying solution until soft enough to cut with a scalpel. The decalcifying solution consists of :

Concentrated hydrochloric acid	4ml.
Glacial acetic acid	3ml.
Chloroform	10ml.
Water	10ml.
Alcohol 97%	73ml.

Each jaw-bone requires about 100ml. of the solution for complete decalcification. The part required for examination

is the root of the incisor in the region of the bend of the jaw just in front of the first molar (Fig. 21). The unneeded parts can be cut away when soft enough, to hasten the decalcification of the rest. The small piece of bone is preserved in 80% alcohol till required for cutting. It is important to cut the section from the same part of the jaw-bone each time, for Fish and Harris (1934) have shown that different parts of the jaw-bone are affected in different degrees.

The details of the preparation of the section are taken from Key's appendix in Goettsch's paper (1928).

(i) *Embedding in gelatin.*—The alcohol is gradually diluted by adding a small quantity of distilled water about every 10 minutes. At the end of half an hour the tooth is transferred to distilled water. This process prevents the tearing of the tissues by sudden changes of surface tension. Meanwhile a test-tube containing a 20% solution of gelatin is heated in a waterbath to 40° C. The piece of tooth is then dropped into the gelatin and the bath is maintained at 35°–40° C. for half an hour. At the end of that time the gelatin, carrying with it the piece of tooth, is poured into a small, shallow dish which has been previously wetted with water. The piece of tooth is arranged with the cut-surface showing the base of the incisor parallel to the surface of the gelatin. The liquid is then left to set. When it is cold a small cube of gelatin, including the tooth, is cut out and placed in a test-tube containing 4% solution of formaldehyde, where it is left for 24 hours to harden.

(ii) *Cutting and mounting the sections.*—Two microscope slides for each tooth are prepared by carefully washing with soap and hot water; then one side of each is coated with a thin layer of Kaiser's glycerin jelly which is prepared as follows:

Forty grams of gelatin are soaked in 210ml. water for 2 hours, then heated with 250ml. glycerine and 5gm. carbolic acid for 10–15 minutes. The solution is filtered through ordinary filter paper at 54° C. On cooling it sets to a jelly. Before use, this is heated on a water-bath until liquid. A very small quantity is spread over each slide with a glass rod. The slides are allowed to stand for 3 hours so that the jelly may set.

The gelatin cube containing the tooth is transferred from the formaldehyde solution to water and left for about half an

hour. The tooth is then cut with a freezing microtome, adjusted to give sections 15μ thick. The sections are placed in a dish of water. Six or eight of them are mounted on each of the prepared slides by means of a glass needle. A clean cigarette paper, supported at the ends by two or three folded papers, is carefully placed over the sections. It is then covered with a thick pad of papers and firmly pressed down. When the papers are removed, the sections remain embedded in the thin layer of gelatin.

(iii) *Staining.*—Two sets of stains are used.

1. Hansen's hæmatoxylin and Eosin.—To prepare Hansen's hæmatoxylin, three solutions are made as follows:

(*a*)	Hæmatoxylin crystals	1gm.
	Absolute alcohol	10ml.
(*b*)	Potash (or ammonium) alum	20gm.
	Distilled water	200ml.
(*c*)	Potassium permanganate	1gm.
	Distilled water	16ml.

The following day solutions (*a*) and (*b*) are mixed, then 3ml. of solution (*c*) are added. The liquid is boiled for 1 minute with continuous stirring, then quickly cooled by placing the vessel containing the liquid in a basin of cold water.

One of the two slides, prepared as above, is placed in this solution for 6–8 minutes. It is then washed in running water for 10 minutes and afterwards left in distilled water for any convenient length of time.

The excess gelatin is wiped off at this stage as it is difficult to clean the slide later.

The process is completed by immersing the slide in:

(*a*) 0·5% aqueous solution of eosin for 1–2 minutes,

(*b*) 96% alcohol for 1½ minutes,

(*c*) fresh 96% alcohol for a further 1½ minutes,

(*d*) absolute alcohol for 3 minutes,

(*e*) xylol for about 2 minutes,

(*f*) fresh xylol for a further 5 minutes.

The cover slips are attached by means of 2 or 3 drops of a solution of Canada balsam in xylol.

2. Hansen's Tri-oxyhæmatin and Connective Tissue Stain.—To prepare Hansen's tri-oxyhæmatin, two solutions are made up as follows:

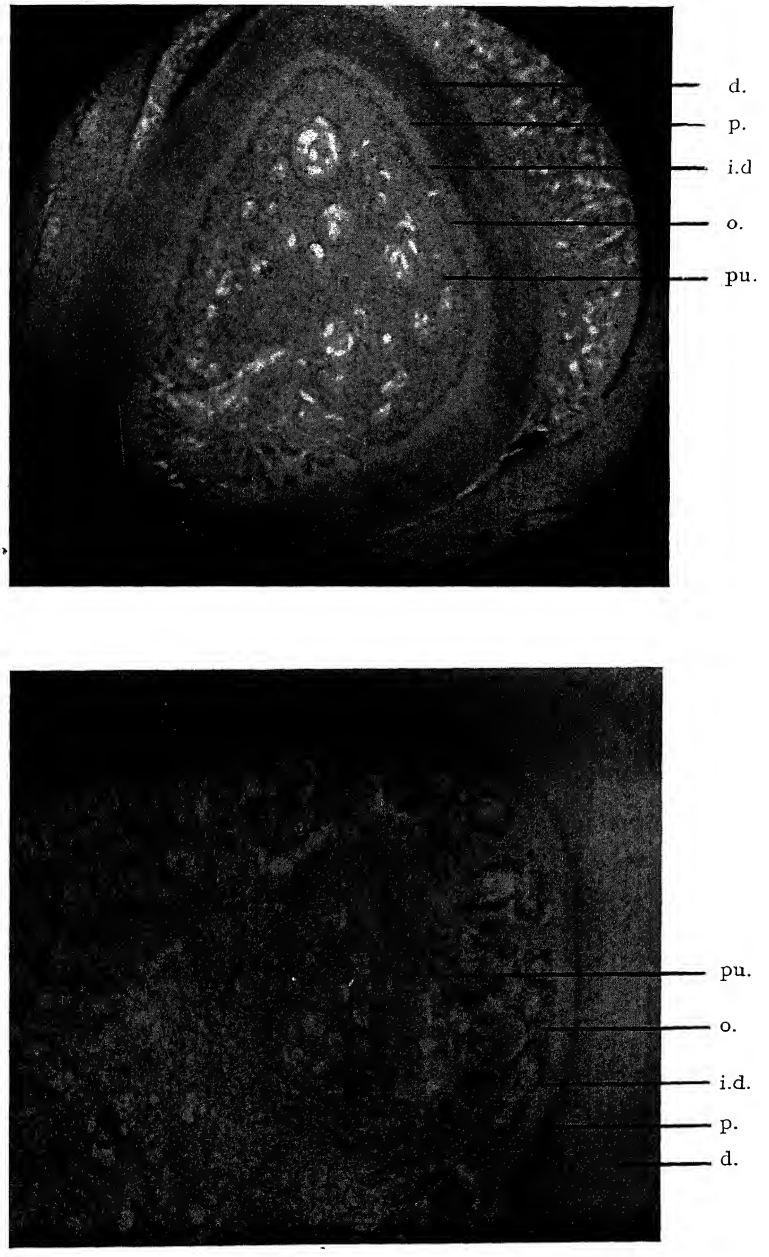

FIG. 17, *a* and *b*.—Amount of protection, o. The amount of protection afforded by vitamin C against histological changes in the structure of the incisors of guinea-pigs brought about in 14 days.

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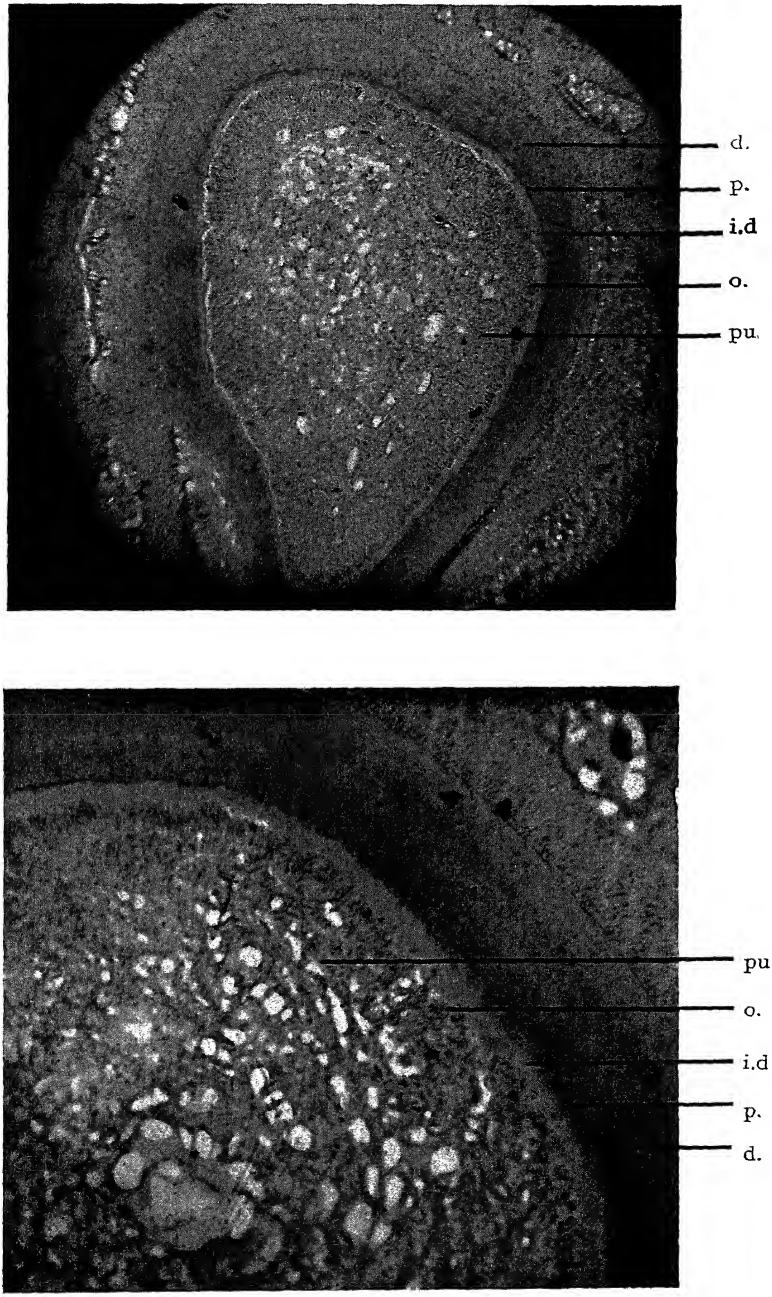
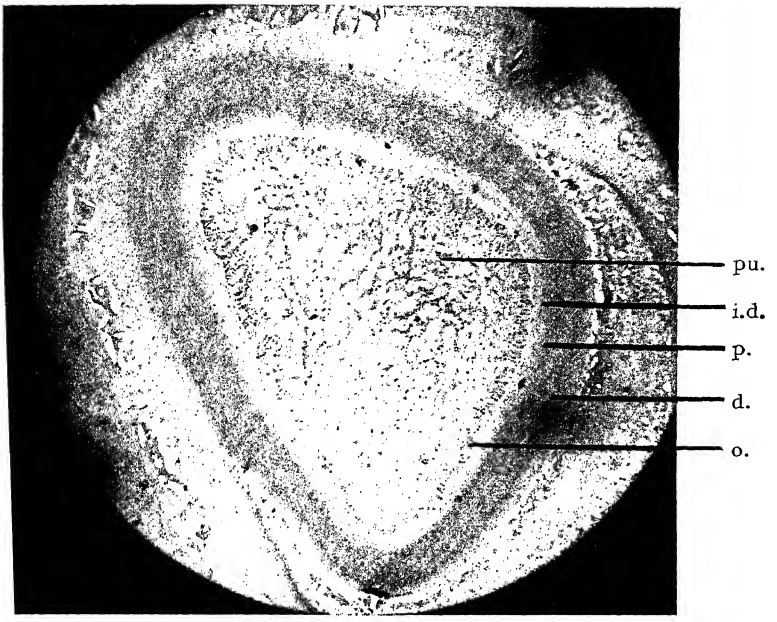


FIG. 18, *a* and *b*.—Amount of protection, 1.



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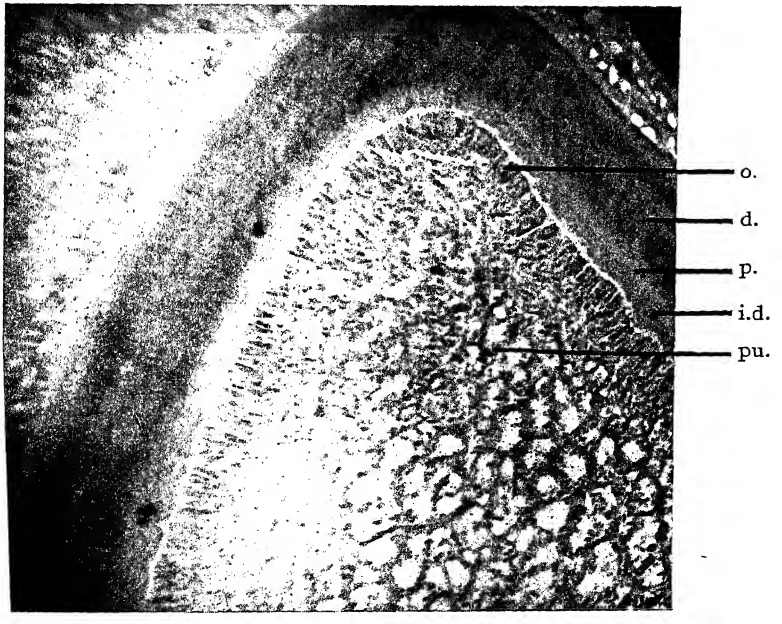

FIG. 19, *a* and *b*.—Amount of protection, 2.

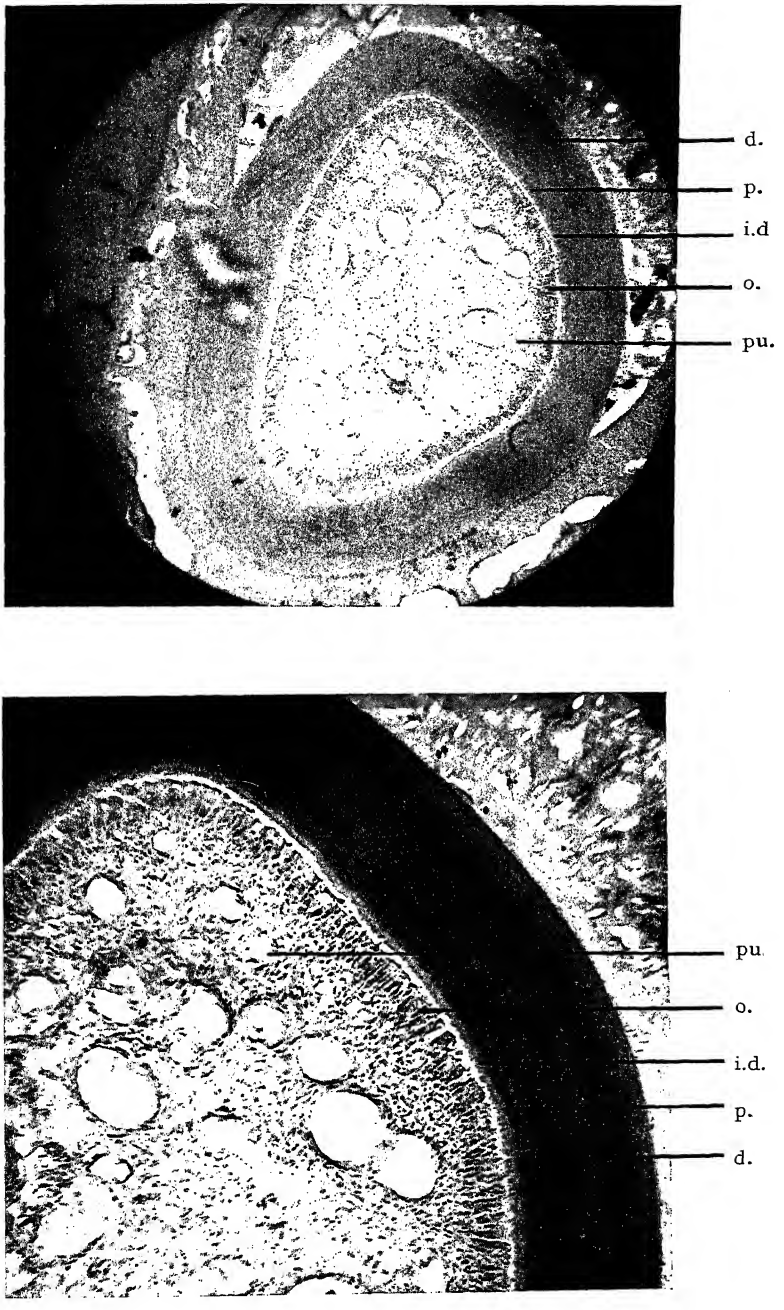

FIG. 20, *a* and *b*.—Amount of protection, 3.

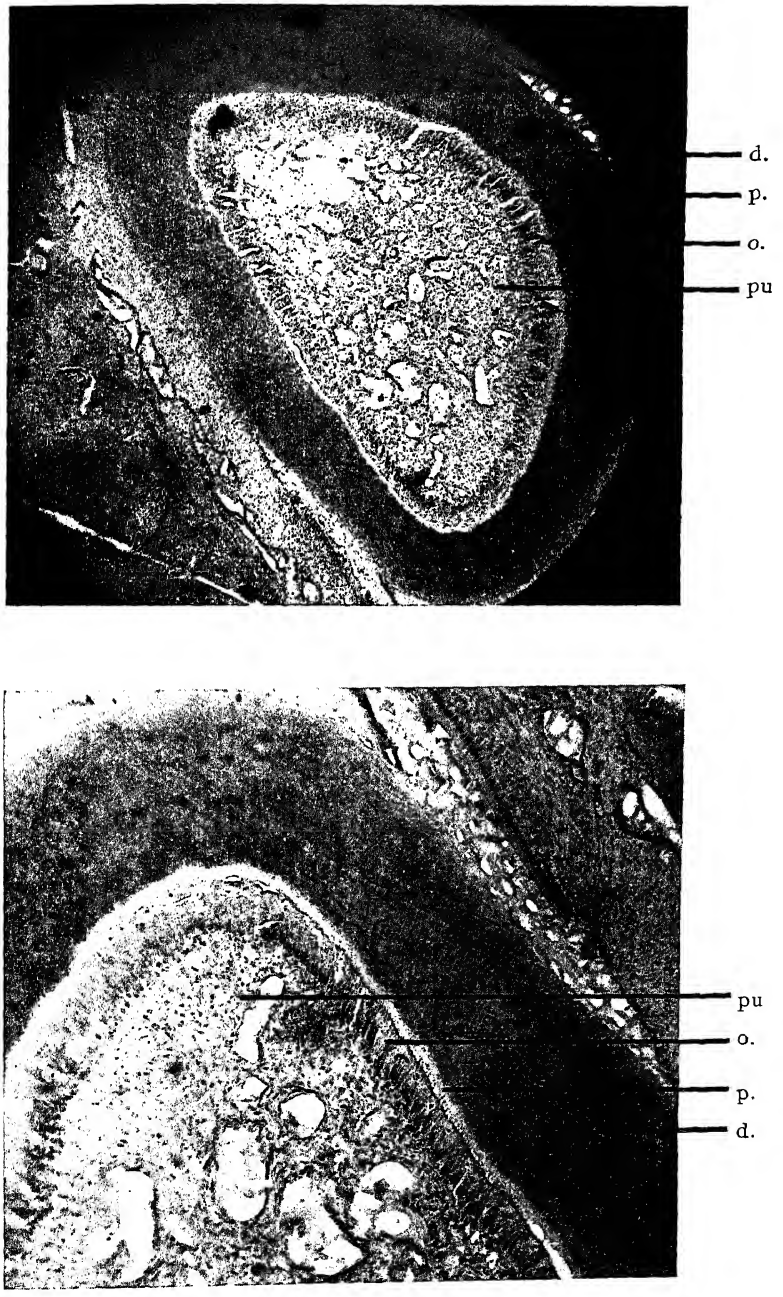

FIG. 21, *a* and *b*.—Amount of protection, 4.

(*a*)	Ferric alum	10gm.
	Distilled water ..	150ml.
(*b*)	Hæmatoxylin ..	1·5gm.
	Warm distilled water	75ml.

Solution (*a*) is poured into solution (*b*) with stirring. The mixture is heated and allowed to boil for half a minute. When cold, the liquid is dark brown with a slight precipitate. It is filtered before use.

The second slide, on which sections have been mounted, is left in this solution for 1 hour, then washed for 10 minutes in running tap water. The excess gelatin is wiped off and the slide placed in distilled water.

Counter-staining is effected with Hansen's connective tissue stain which is prepared as follows:

100ml. of saturated aqueous solution of picric acid is mixed with 5ml. of 2% aqueous solution of acid fuchsin. This mixture is kept as a stock solution and 7 drops of 1% acetic acid solution are added just before use.

The slide is kept in this dye for 20–30 minutes, then rinsed in acidulated water (30ml. distilled water and 20 drops of 1% acetic acid solution) for a few seconds. The sections are dehydrated in alcohol and cleared in xylol and the cover-slips are attached with Canada balsam as described above.

(*c*) *Working out the result.*—In order to average the results given by the different members of any group of guinea-pigs, all of which have been given the same daily dose, each tooth section must be compared with a scale consisting of a series of sections showing graded degrees of protection which have been given numerical values corresponding to the degree of protection shown. Such a scale (Figs. 17–21) was drawn up by Key and Elphick (1931). The cross-section of a normal tooth consists of pulp in the middle surrounded by a layer of long parallel odontoblasts, a narrow predentine and a wider dentine. Fine Tomes' canals run from the odontoblasts through the predentine and dentine. In a scorbutic tooth, the odontoblast layer is disorganised, the predentine is calcified and an irregular layer of bone is formed between the predentine and the odontoblasts. The Tomes' canals are fewer and are only found radiating from the predentine outwards. The extent of the disorder of each part varies with the smallness of the dose of vitamin C given, but

with a particular dose, the disorder of all parts is not always greater than the disorder of all parts in a pig given a larger dose. Thus an average value for a tooth has to be assigned from a consideration of all parts that may be disordered. Key and Elphick found the scheme in Table VII useful for evaluating the amount of protection from scurvy afforded by a dose of vitamin C, the figure 0 being given to the severest form of scurvy developed in 2 weeks by feeding pigs on a completely scorbutic diet, and the figure 4 being given to a state of complete protection from scurvy.

TABLE VII

SCHEME FOR ASSESSING THE SEVERITY OF SCURVY DEVELOPED IN GUINEA-PIGS IN 14 DAYS WHEN GIVEN A DIET PARTLY OR WHOLLY DEFICIENT IN VITAMIN C

Odontoblasts.	Inner dentine.	Predentine.	Tomes' canals.	Degree of protection from scurvy.
Disorganised	Wide, with projections into pulp	Calcified	Only in outer dentine	0
Completely disorganised in places, but some parallel formation	Narrow and irregular	Calcified	Only or mostly in outer dentine	1
All parallel but becoming disorganised near pulp	Narrow	Calcified or partly calcified	Mostly in outer dentine or cross inner dentine from odontoblasts	2
Long and parallel but becoming disorganised near pulp	Absent or mere rim	Not calcified	Cross dentine from odontoblasts	3
Long and parallel	Absent	Not calcified	Cross dentine from odontoblasts	4

Thus by using a scheme such as this a numerical value can be given to each tooth. The values for all the guinea-pigs in a group can be averaged and the averages of the different

groups, given different doses, can be compared. Whatever may have been the arrangement of doses chosen, this method of evaluating results may be used.

Example.—A certain concentrate was examined in the following way : 30 guinea-pigs were divided into five groups of 6 animals each. All the pigs in any one group were given the same dose ; four of the different groups were given daily doses of 0·4mg. ascorbic acid (not the International Standard), 0·8mg. ascorbic acid, 0·75ml. diluted concentrate and 1·5ml. diluted concentrate respectively, and the fifth group was given no dose. The result of the examination of the teeth and evaluation according to the scheme in Table VII was summarised as follows :

Dose.	Protection afforded to each guinea-pig.						Average protection afforded to the group.
0·4mg. ascorbic acid	1·5	0·5	2·0	1·0	1·0	2·0	1·33
0·8mg. ascorbic acid	2·0	3·5	1·5	2·5	1·5	1·5	2·08
0·75ml. diluted concentrate	1·5	0·5	1·0	1·0	1·5	1·0	1·08
1·5ml. diluted concentrate	3·0	1·0	3·5	2·5	3·5	3·0	2·75
No dose	1·5	1·5	1·0	0·0	0·0	1·0	0·83

By a simple comparison of the amount of protection afforded by each dose given to the different groups of guinea-pigs, it is evident that :

(i) 0·4mg. ascorbic acid has afforded a little more protection than 0·75ml. of the diluted concentrate ; thus 0·75ml. of the diluted concentrate appears to be a little less potent than 0·4mg. ascorbic acid.

(ii) 0·8mg. ascorbic acid has afforded a little less protection than 1·5ml. of the diluted concentrate ; thus 1·5ml. of the diluted concentrate appears to be a little more potent than 0·8mg. ascorbic acid.

From the two comparisons it may be concluded that 1·0ml. of the diluted concentrate is equivalent to about 0·5mg. of the ascorbic acid with which it was compared. (Note.—As this was not the actual sample of ascorbic acid which is in use as the International Standard, the result cannot be expressed in International Units.)

The figures given in column 2 of the list of results of this experiment indicate the amount of variation that may be expected between different animals.

(*d*) *Use of a curve of response.*—If it is anticipated that many tests for vitamin C will have to be made it will save labour and animals if a preliminary experiment is made to construct a curve of response. This should be made with graded doses of the International Standard if possible, though Key and Elphick constructed one with graded doses of orange juice before the International Standard preparation of ascorbic acid was available. This, however, is immaterial since the curve of response is only used for comparing the average results from two groups of animals, and never for obtaining a value corresponding to the average result from one group only.

The more animals used for testing each dose of Standard the smoother will be the curve of response. Key and Elphick found that 15 animals on each of four doses of orange juice gave a very satisfactory curve. Possibly 10 on each dose would give a satisfactory result also. It is desirable, of course, to begin dosing all the animals on the same day. If this is not possible, a smaller number of animals may be started at different times, provided that each number is a multiple of 4 and that equal numbers of pigs started at any one time are given each of the four doses chosen. The average results for the different groups may then be obtained, and plotted against the doses given to obtain the curve of response relating protection from scurvy to dose of vitamin C given.

The curve obtained by Key and Elphick proved to be a straight line (Fig. 15). It is interesting to note that the line does not pass through the origin of the graph ; that is, in the guinea-pigs given no dose of vitamin C, the average intensity of scurvy produced in the 2 weeks' period of feeding with no vitamin C, was not as severe as the stage to which the value 0 had been assigned by these workers. The curve of response obtained after, say, 3 weeks of feeding instead of 2 could be constructed if desired, but severer symptoms of scurvy than those to which the value 0 had been given would have to be recognised and designated by values of −1, −2, etc.

When the curve has once been constructed it is necessary to test only one dose of the test substance against one dose of Standard, provided there is some information available as to

the possible potency of the test substance. The ratio of the potency of the test substance and dose of Standard are, then, not the ratio of the average amounts of the protection afforded by them, but the ratio of the abscissæ of the curve corresponding to the amounts of protection afforded by those doses.

Example.—Suppose:

(*a*) a dose of 3ml. fruit juice afforded an average amount of protection of 3·2, and

(*b*) a dose of 0·5mg. ascorbic acid (International Standard) afforded an average amount of protection of 1·5.

The abscissa of the curve of response corresponding to 3·2 degrees of protection was 2·375.

The abscissa of the curve of response corresponding to 1·5 degrees of protection was 0·8.

Thus the ratio of the potencies $\frac{\text{3ml. juice}}{\text{0·5mg. A.A.}}$ is not $\frac{3{\cdot}2}{1{\cdot}5}(=2)$ but $\frac{2{\cdot}375}{0{\cdot}8}$ which is 3; whence 3ml. juice would contain $3 \times 0{\cdot}5$mg. ascorbic acid and the juice would contain 0·5mg. ascorbic acid or 10 International units of vitamin C per gram. The use of a curve is obviously not necessary when the results happen to be close together, but when they are far apart, as in the example given, the curve is useful.

B. Increase in weight.

The influence of vitamin C on the increase in weight of guinea-pigs has been established by Bracewell, Hoyle and Zilva (1930). In their report on the "Anti-scurvy Vitamin in Apples" they say that in assessing the biological results quantitatively, one has to consider several independent factors, namely, duration of test period, cause of death, first appearance of scorbutic symptoms, degree of scurvy at autopsy, and growth. They have published thirty-three charts of growth curves of animals. Each chart represents the test on 10–24 guinea-pigs in three or four groups, the groups having been given different doses of one sample of apple and all the pigs in any one group having been given the same dose. The curves of growth of the animals of any one group are drawn in the same diagram. Thus it is easy to see how far the different animals have varied in their response to a particular dose of a

particular kind of apple and also to get a general idea of how far different groups of animals varied in their response to different doses of the same sample of apple. The test was carried on for 60–90 days. The diet used has already been described in this chapter. The doses tested were generally 3, 5, 10 and 20g. respectively. No simultaneous tests on a standard of reference were made; comparisons between different samples were made from results obtained at the same or at different times simply from the animal reactions. Thus no consideration was given to possible differences in sensitivity of the guinea-pigs at different times and no allowance was made for the possibility.

The charts, however, demonstrate the following points:

(*a*) Given a small dose of vitamin C (in apple), most guinea-pigs will grow for about 3 weeks but will then cease to grow and may lose weight and die.

(*b*) Given a rather larger dose, the same initial increase in weight will be observed, and it may be followed by a long period of neither increase nor decrease in weight.

(*c*) Given a larger dose still, increase in weight will continue till the pigs reach a weight of 500g. or more.

If vertical lines are drawn through the points representing the end of 4 weeks of these tests, the following observations may be made:

(*a*) The variation in result in all the groups is very great.

(*b*) There seem to be three types of results distinguishable at this stage:

(1) Loss of weight.
(2) Maintenance of weight.
(3) Increase in weight, which is curiously about the same rate in all the groups that show increase in weight at all in each chart.

The difference between loss of weight and maintenance of weight is attained by doubling the dose. The difference between maintenance of weight and increase in weight is attained by doubling the dose. Redoubling the dose again does not increase the rate of growth any further.

This indicates that the curve of response relating increase in weight of guinea-pigs to dose of vitamin C given is very steep which indicates that this should be an accurate method of determination of vitamin C.

Coward and Kassner (1936), recognising the simplicity of the "increase in weight" method, constructed a curve of response relating increase in weight of guinea-pigs in 6 weeks to dose of vitamin C given. As was foreseen from Zilva's results

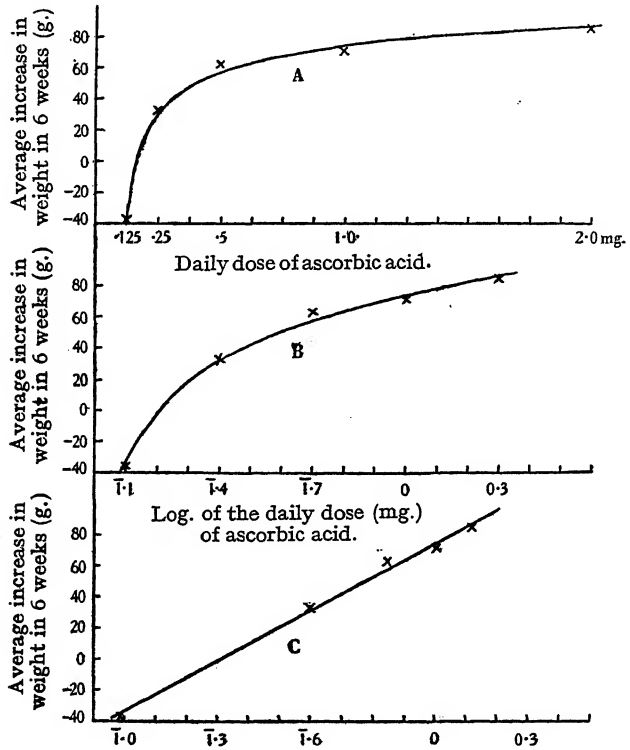

FIG. 22.—The mean increases in weight of groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) the dose of ascorbic acid (x) (equation $y=74{\cdot}3+108{\cdot}2$ log. (log. $10x$));

(B) the log. of the dose of ascorbic acid (log. x) (equation $y=74{\cdot}3+108{\cdot}2$ log. $(x+1)$);

(C) the log. of the log. of ten times the dose of ascorbic acid log. (log. $10x$) (equation $y=74{\cdot}3+108{\cdot}2x$));

to show how the equation of the curve of response was obtained.

the curve was a very steep one (Fig. 22), but as shown in Part II Chapter XI, the accuracy obtainable by its use is about the same as that obtainable in the "tooth" method. The curve of response to vitamin C is used in the same way as other curves of response. Two groups of animals are used, each animal of

one group being given a daily dose of the Standard and each animal of the other group being given a daily dose of the substance under examination. If no information of the possible potency of the substance is available a preliminary test with two or more doses covering a wide range must be made. The average increases in weight of the groups are not directly proportional to the vitamin content of the doses given, but to the abscissæ of the curve of response corresponding to these increases. Therefore the abscissæ corresponding to the increases in weight of the groups are found and a proportion sum worked out to obtain the potency in International units of the substance examined.

C. **Comparison of the two methods.**

The great advantages of the "tooth" method over the "increase in weight" method are (1) that it is specific for vitamin C, and (2) that the time required for the "tooth" method is shorter than the time required for the "increase in weight" method.

The advantages of the "increase in weight" method over the "tooth" method are (1) that it requires no preparation of sections for histological examination, and (2) that the assessment of the response is made from the weight of the animal and not from an examination of the derangement of the tooth structure.

The two methods give results of approximately equal accuracy. Thus the choice of method must depend on the skill available for the carrying out of the determination. If histological technique can be used, there can be no doubt that the "tooth" method, being specific for vitamin C, is far superior to the "increase in weight" method which is not specific for vitamin C.

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CHAPTER VI

THE DETERMINATION OF VITAMIN D

1. The International Standard of Reference and the Unit of Vitamin D Activity.
   - A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin D is made.
   - B. The dilution of the Standard of reference for dosing.
   - C. The general arrangement of the test for a determination of the vitamin D potency of a substance in terms of the International Standard.
2. The Preparation of Rats for a Determination of Vitamin D.
   - A. Animals suitable for the test.
   - B. Housing of the animals.
   - C. Rachitogenic diets.
3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin D.
   - A. The " line " test, a curative method.
   - B. The " X-ray " method, generally curative.
   - C. The " bone-ash " method, generally prophylactic.
   - D. The " increase in weight " method.
   - E. Comparison of the four methods.
4. The Difference in Results obtained by using (*a*) Rats and (*b*) Chickens in the Determination of Vitamin D.
5. References.

VITAMIN D promotes the calcification of bone and the formation of hard dentine and enamel in teeth. When the ratio of calcium to phosphorus in the diet is unity less vitamin D is necessary than when the ratio of these elements is greater or less than unity. A recent summary of the evidence on this point, together with fresh experimental evidence, has been made, in English, by Querido (1935). The ratio Ca : P=4 : 1 generally produces rickets in rats which have low reserves of vitamin D, and hence it is useful for experimental purposes. The existence of more forms than one of vitamin D is discussed on p. 127.

1. The International Standard of Reference

The International Standard of Reference for vitamin D is a solution of irradiated ergosterol in olive oil kept at the National Institute for Medical Research, London.

The International unit of vitamin D is the activity of 1mg. of the International Standard of Reference.

This is the unit which was recommended for adoption by the Permanent Commission for Biological Standards of the League of Nations in 1931. It was not modified in any way when the standards were reconsidered in 1934. It is the unit that the Medical Research Council of Great Britain had adopted in 1930 and it is also the unit that the Pharmaceutical Society of Great Britain had used since 1927.

A. **The need for a simultaneous test on the Standard of reference whenever a determination of vitamin D is made.**

The severity of the rachitic condition developed in young rats by feeding them on a diet which is deficient in vitamin D and which has a high-calcium, low-phosphorus content depends on many factors, the chief being:

(i) the rats' reserves of vitamin D, and

(ii) the length of time during which the rachitogenic diet is given to the rats, *i.e.* the preparatory period.

The more severe the rachitic condition of the rats the less is their response to a particular dose of vitamin D. To gain some idea of the extent of the influence of the length of the preparatory period on the amount of healing produced by a particular dose of vitamin D, an experiment was carried out in the writer's laboratory. Five litters of rats consisting of 6, 8, 4, 4 and 8 rats respectively were divided into two groups, one-half of the animals of each litter being assigned to one group and the other half to the other group. The preparatory period of one group was 21 days and of the other group 27 days. Each animal was given 5 units of vitamin D at the end of its preparatory period, and kept on test without further supplement for 10 days. Healing was assessed by the "line" test as carried out in that laboratory (described later in this chapter). The average healing of the rats which were given the dose of vitamin D after 21 days' preparation was 3·2 (on a scale in which 6 represented complete healing) and the average healing of the rats which were given the dose of vitamin D after 27 days' preparation was 2·4. Thus the influence of the length of the preparatory period on the amount of healing brought about by a dose of vitamin D is appreciable.

It must never be assumed that rats which have apparently

received the same treatment have developed the same sensitivity to vitamin D, and a simultaneous test must be made on the Standard of reference whenever the vitamin D content of a

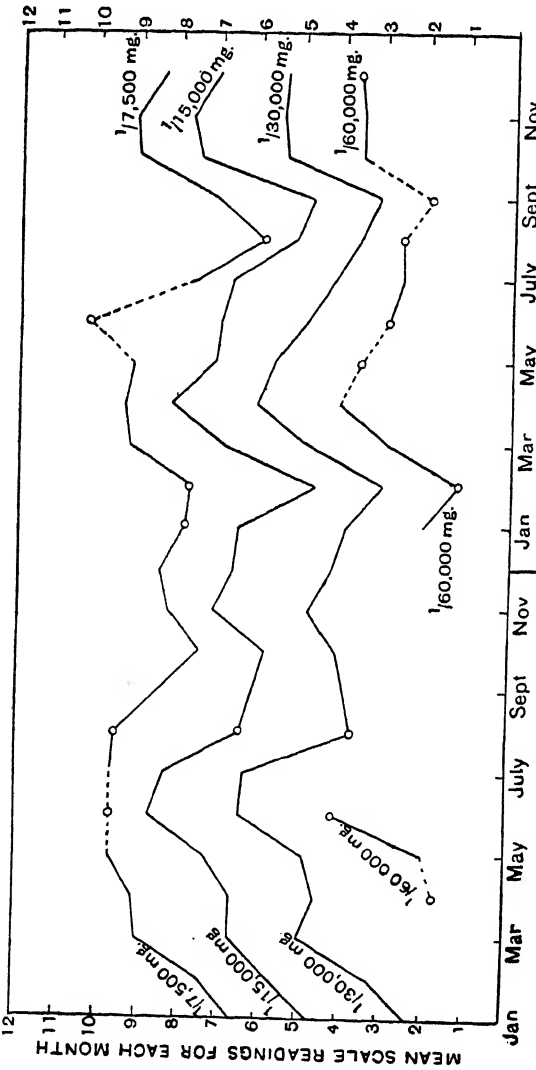


FIG. 23.—Variations in degree of healing caused by constant doses of vitamin D (irradiated ergosterol) in successive months. The average number of rats for each point plotted is 19. Points for which less than 10 rats were available are surrounded by circles. The dotted portions of the curve refer to points for which only 3, or 4, rats were available.

substance is determined. The fluctuations in the response of the rats from one colony to the same dose of vitamin D (International Standard) have been shown by Bourdillon, Bruce, Fischmann and Webster (1931) (Fig. 23), and a similar

result from another colony has been shown by Coward and Key (1933). The latter has been brought up to date in Fig. 24. All the rats had been subjected to the same treatment as far as could be determined and yet the healing brought about by

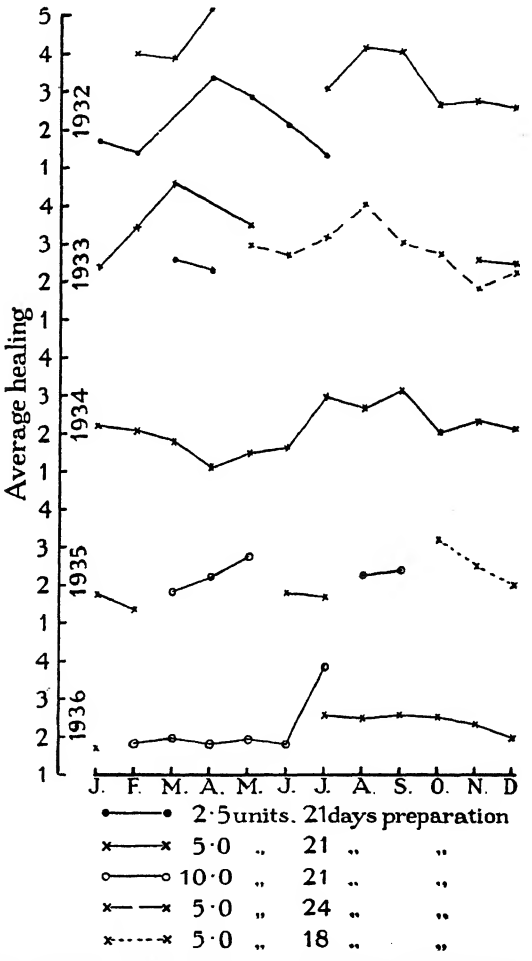


FIG. 24.—Fluctuations in the response of rats to vitamin D from month to month.

the same dose of the Standard at different times varied so much that at one time it was more than twice as great as at another time. The figures were averages of large numbers of rats and the fluctuations were gradual but not, apparently,

seasonal. Thus the differences in response were not merely the differences one expects to get between individual rats, but were apparently due to some other influence over which the workers had no control.

It is evident that a sample of cod liver oil examined simply by its influence on a group of animals would produce different amounts of healing at different times in the same laboratory, and its vitamin D content would therefore appear to vary from time to time. It might be expected that this difficulty could be overcome by examining the rats' bones by X-ray just before dosing with the cod liver oil and always using only those rats which appeared to have rickets of equal severity. But that was exactly the procedure followed by Bourdillon *et al.* and yet they obtained the fluctuations described. In Table VIII may be seen examples of the variation in average response of groups of 10 rats obtained in the writer's laboratory and the gradual fluctuations of the response throughout several months.

Thus the only possible way to use the Standard of reference for vitamin D is to divide each litter of rats available for a determination into two groups as nearly alike as possible and to use one group for dosing with the International Standard and the other group for dosing with the substance whose vitamin D potency is to be determined.

It follows from the above that it is completely wrong to examine the effect of a dose or of several doses of the International Standard on the rats of any colony, however uniform it may appear to be, and then to compare results obtained subsequently with the results previously obtained on the International Standard. This does *not* give a result in terms of the International Standard. That can only be obtained by making a simultaneous test on the Standard whenever a determination of the vitamin potency of a substance is made.

B. **The dilution of the Standard of reference for dosing.**

Since the International Standard of reference is a solution of irradiated ergosterol in olive oil of such a strength that 1 mg. of the solution contains 1 International unit of activity, it is necessary to dilute this solution before giving it to the rats, for the dose to be given is so small that the difficulty of giving it accurately would introduce a large error into the method. The dilution should be made by weight and should be of such

TABLE VIII

VARIATION IN THE AVERAGE HEALING OF GROUPS OF RATS WHICH, HAVING BEEN GIVEN THE STEENBOCK, 2965, RACHITOGENIC DIET FOR 21 DAYS, WERE THEN GIVEN 5 INTERNATIONAL UNITS OF VITAMIN D IN ONE DOSE AND NO FURTHER SUPPLEMENT FOR 10 DAYS

1936.												1937.			
JULY.		AUGUST.		SEPTEMBER.		OCTOBER.		NOVEMBER.		DECEMBER.		JANUARY.		FEBRUARY.	
No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.	No. of rats in group.	Average healing.
11	2·95	10	2·50	9	1·83	9	2·56	10	2·60	16	2·06	12	2·00	9	2·28
10	3·17	10	2·15	10	1·70	9	2·33	10	2·45	10	2·20	11	1·77	10	2·00
11	2·86	13	2·42	10	2·05	9	2·44	10	2·25	15	1·83	10	1·85	10	1·55
9	2·17	10	2·15	12	2·50	9	2·83	10	2·15	10	1·65	10	2·15	10	1·50
9	2·50	10	2·45	12	2·29	9	2·55	9	2·33	9	1·89	9	1·77		
9	2·56	10	3·20	12	2·71	10	2·85	10	2·25	10	2·25	10	1·85		
21	1·93			8	2·44	10	2·50	10	2·55	10	2·25	20	1·65		
				10	2·85	10	2·45	10	2·15	15	1·80				
				10	2·90	10	2·65	10	2·25						
				15	2·83	10	2·15	10	2·40						
				11	2·82	10	2·10	9	2·22						
				6	3·00	9	2·06	10	2·30						
				12	2·50	9	3·56	10	2·40						
				10	2·90	9	2·22	10	2·40						
				11	2·68	10	2·30								
				10	2·95	10	2·60								
				10	2·95										
				10	2·75										
Weighted means	2·56		2·48		2·59		2·51		2·34		1·98		1·84		1·82

a concentration that the amount to be given to each rat is contained in whatever weight or volume can be conveniently and accurately given to the rats. For doses as small as 10mg. (and often for 20mg. or 50mg. also) the writer uses the Burroughs Wellcome " Agla " syringe fitted with a blunt injection needle which can be held in a rat's mouth by one worker while another gives the turn of the screw required for delivering the dose of oil or solution. The volume of the dose has, of course, to be adjusted to the specific gravity of the oil. An average value, 0·925, for ordinary cod liver oils can be used, and an average value, 0·92, for olive oil can be used for the diluted Standard.

C. **The general arrangement of the test for a determination of the vitamin D content of a substance in terms of the International Standard.**

It has already been shown in the earlier part of this chapter that a simultaneous test on the Standard must be made whenever the vitamin D content of a substance is to be determined. It was mentioned that half of the rats of each litter used should be given doses of the Standard and the other half should be given doses of the test substance. This is necessary because the reserves of vitamin D in rats differ from litter to litter ; and differences in reserves bring about differences in the severity of rickets developed and differences in the amount of healing after dosing with the same amount of vitamin D. As there is no method of determining the rats' reserves, the only precaution that can be taken is to divide each litter into two groups as nearly alike as possible and use one group for the Standard of reference and one for the test substance. This, of course, applies to both curative and prophylactic tests.

If an occasional test only is to be made, the rats allocated to the Standard are divided into three groups and the groups given different doses of the Standard in the ratio 4 : 2 : 1, each rat in any one group receiving the same dose. The rats allocated to the substance under examination are similarly divided into three groups for the testing of three different doses of the test substance in the ratio 4 : 2 : 1 (or, if no information is available of the probable potency of the test substance, in a wider range, say, 9 : 3 : 1). Each of these six groups should contain at least 6 animals. When the results from the different groups are averaged, it may be found that

the results from the three groups given doses of the Standard in the ratio 4 : 2 : 1 correspond with the results from the three groups given doses of the test substance in the ratio 4 : 2 : 1 when good evidence of the potency of the test substance would be obtained. It may happen that the results from the two higher doses of test substance are similar to the results from the two lower doses of Standard respectively, which would also give satisfactory evidence of the potency of the test substance. If, however, one dose only (say, the highest dose) of test substance corresponded to one dose only of the Standard, the lowest dose, then, in view of the variation in animal response, this evidence would not be very good. A further test should be made with doses which, judging from the first test, are more nearly equal to the doses of Standard, the same doses of Standard being used again in the second test. This arrangement of the animals may be used whatever the criterion adopted for the measurement of the response to vitamin D and whether the test is made as a curative or as a prophylactic one.

If many determinations by the curative method are contemplated, an economy in labour and animals is effected if a curve of response to graded doses of vitamin D is first constructed. It can then be used for comparing the average results from two groups of rats, one of which has been given a dose of the Standard and the other a dose of the substance under examination, some knowledge of its probable potency having been previously obtained. The curve is constructed in a way very similar to that used for constructing a curve of response to vitamin A. Ten litters of rats should be used. After the preliminary period of feeding on a rachitogenic diet the rats are placed in separate cages. One rat of each litter is given, say,

(*a*) no dose,
(*b*) 0·125 unit vitamin D daily,
(*c*) 0·25 unit vitamin D daily,
(*d*) 0·5 unit vitamin D daily,
(*e*) 1·0 unit vitamin D daily,
(*f*) 2·0 units vitamin D daily for the period of the test, or a single dose ten times the amount of the daily dose may be given and no further dose for 10 days. The results are averaged and plotted against the doses of vitamin D given to construct the curve of response (Fig. 25).

The curve of response to vitamin D is used in the following way: When some idea of the potency of the test substance is available, one dose of it is tested against one dose of the Standard, the doses being chosen to give as nearly the same result as possible. Then half of each litter available is given the dose of test substance and the other half is given the dose of Standard. Eight to ten rats should be used for each. The results obtained from the dose of test substance and Standard respectively are averaged. The averages are not compared directly, but through the curve of response. The abscissa

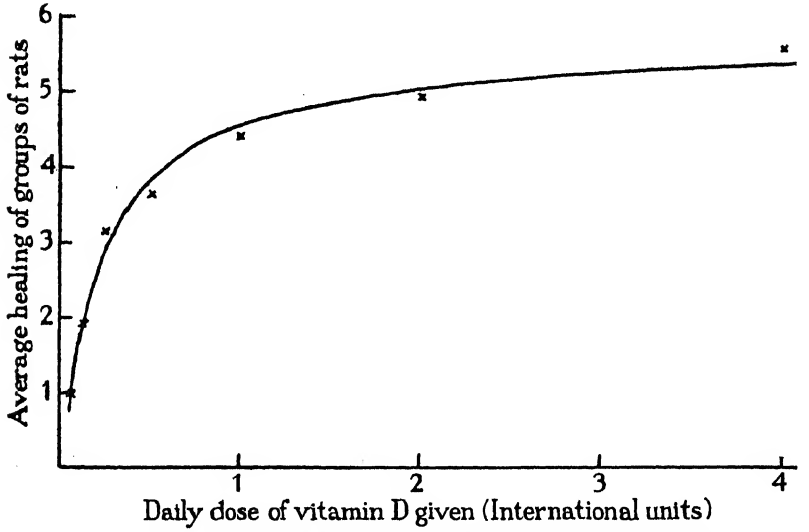


FIG. 25.—Curve of response relating healing of rats to dose of vitamin D given.

corresponding to each average result is found from the curve. The relative potency of the two doses is then the ratio of the two abscissæ corresponding to the two average results, and the potency of the test substance is calculated in terms of the Standard.

It must never be assumed that a curve of response to vitamin D constructed in one laboratory can be used for the interpretation of results obtained in any other laboratory. Dyer (1931) and Key and Morgan (1932) have shown that rats of the same colony that developed different degrees of rickets under the same treatment gave very different curves of

response to the same graded doses of vitamin D (Fig. 26). Rats from different colonies might behave more differently still.

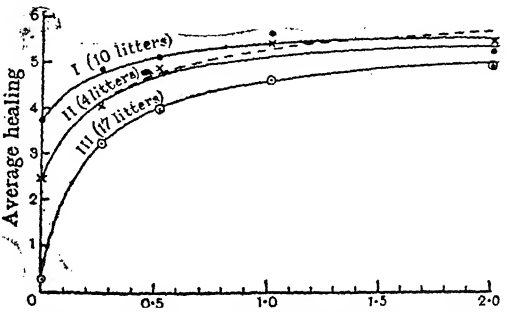


FIG. 26.—Different curves of response to vitamin D from rats having different degrees of rickets at the beginning of the test period, *i.e.* when the vitamin D was first given.

A curve of response can only be used for animals brought to a condition very similar to that of the animals used in the construction of the curve. Just as no curve of response can

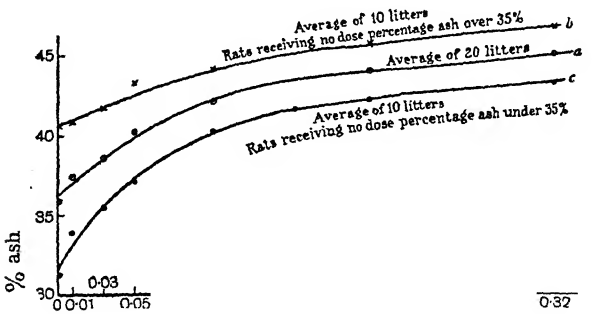


FIG. 27.—Curves of response relating the percentage ash in dry fat-extracted bone to dose of vitamin D given (Hume, Pickersgill and Gaffikin). Different curves of response are obtained according to the percentage ash in the rats which received no supplement to the rachitogenic diet.

be used in a prophylactic test for vitamin A, so none can be used in a prophylactic test for vitamin D. This has been very clearly shown by Hume, Pickersgill and Gaffikin (1932) for the " ash content of the bone " method (Fig. 27).

2. The Preparation of Rats for a Determination of Vitamin D

A. Animals suitable for the test.

The colony of rats which is to supply animals for vitamin D work should be fed on a diet containing enough vitamin D for growth and reproduction but not enough to make the reserves of the young so large that they cannot develop rickets when given a rachitogenic diet. The feeding of a colony of rats that produces young suitable for vitamin D work has been described in Chapter II. Young rats from this colony are generally suitable to begin their preparation for a vitamin D determination by the curative method at a weight of 50–60g., but this will vary somewhat according to the condition of the colony. For a prophylactic test, rats of a lower weight (about 40g.) are generally used.

B. Housing of the animals.

The laboratory in which vitamin D work is to be carried out should receive no direct sunlight. If it does, blinds should be provided and kept drawn while the sun is shining directly on the windows. Even "sky-shine" without direct sunlight has been found to cure experimental rickets.

All the rats of one litter may be kept together in one cage of dimensions about 12″ × 18″ × 8″ during the preparatory period. During the period of dosing (often called the "test period") each rat should be kept in a separate cage of dimensions about 9″ × 12″ × 6″. Bedding may be of sawdust or shavings, and provided that this has not been exposed to strong sunlight or radiations from a carbon arc, wire screens are not necessary. Cages should be cleaned thoroughly once a week.

C. Rachitogenic diets.

Steenbock's rachitogenic diet, No. 2965 (Steenbock and Black, 1925), has been found satisfactory in a great many laboratories. It consists of:

Yellow maize, whole seed ground finely	76%
Wheat gluten	20%
Calcium carbonate	3%
Sodium chloride	1%

McCollum's diet, No. 3143 (McCollum, Simmonds, Shipley and Park, 1921), has also been found satisfactory in many laboratories. It consists of:

Whole wheat, ground	33%
Yellow maize, whole seed ground finely	33%
Gelatin	15%
Wheat gluten	15%
Calcium carbonate	3%
Sodium chloride	1%

It is absolutely essential that the constituents of these diets should be thoroughly mixed, for the development of rickets depends on the calcium-phosphorus ratio of the diet and it would be fatal to the success of the experiment if some rats ate more calcium carbonate than the others. It is therefore advisable to mix not more than 3 kilos. of diet at a time, mixing first the sodium chloride and calcium carbonate by rubbing together in a mortar, then adding the wheat gluten in two to three portions, then the yellow maize and other constituents, if any.

Water.—Some workers supply their rats with distilled water, and this may be necessary in some districts. The London tap water (notably a "hard" water) has been found suitable. It should be given fresh each day in an inverted flask or bottle provided with a straight outlet tube which lets out drops of water only when a rat licks the lower end. The drinking water is thus kept clean.

3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin D

The criterion usually employed for the determination of vitamin D is the calcification of bones of rats which have been rendered suitably rachitic by feeding them on a diet which has a high-calcium, low-phosphorus content and which is devoid of vitamin D. This reaction is almost specific for vitamin D. The only method other than the giving of vitamin D of bringing about improved calcification after a diet with a high-calcium, low-phosphorus content has been used for some time, is to reduce the Ca : P ratio to a value more nearly equal to 1 (Key and Morgan, 1932; Bruce and Callow, 1934). The presence of a high percentage of phosphorus in the substance under examination would therefore improve the calcification of the bones of the test animals independently of the action of vitamin

D which might also be present. The vitamin D value of the substance would then appear to be higher than it really was and another determination of its potency in which a diet containing a different Ca : P ratio was used would fail to confirm the former result. This difficulty is not encountered in the determination of vitamin D in cod liver oil and concentrates, but whenever a large dose of a relatively weak substance has to be used for estimating the vitamin D content a determination of the phosphorus content should first be made and, if necessary, allowance then be made for it in calculating the result; or the diet of the control rats should have the Ca : P ratio adjusted to equal that of the rats receiving a dose of the test substance. This can easily be done by substituting the required amount of calcium phosphate for part of the calcium carbonate in the diet.

The growth-promoting (increase in weight) property of vitamin D was first demonstrated by Steenbock, Nelson and Black (1924). How far this property could be used for the determination of the vitamin was investigated by Coward, Key and Morgan (1932), who demonstrated a curvilinear (logarithmic) relationship between the dose of vitamin D given and the increase in weight of rats which were given different doses of vitamin D daily for 5 weeks after they had become steady in weight on a diet deficient in this factor (Fig. 33). The individual variation of the response of rats to the same dose of vitamin D was found to be much greater than that of rats in the corresponding vitamin A test. There was therefore no temptation to substitute this test for the more specific one in which the promotion of calcification was the criterion used.

A. The " line " test, a curative method.

This test requires a preparatory period during which all the animals are given a rachitogenic diet *ad lib.* followed by a curative period during which doses of Standard and test substance are given.

(i) *Preparatory period.*—Two and a half to three and a half weeks will in general be sufficient to produce severe rickets in rats fed on Steenbock's diet, 2965, provided the diet of the stock colony is poor in vitamin D. This condition can be detected by the swollen condition of the wrists and knees. A halting gait may be detected.

The rats should be weighed once a week during this time. If any lose more than 2–3g. weight they should be discarded. Generally the rats grow, but at a rather slow rate.

The diet should be supplied in abundance. No record of the amount eaten need be kept.

(ii) *Curative period.*—The length of the curative period generally adopted is 10 days. The dose of Standard and of unknown may be given daily throughout the test period, or if the total amount for the 10 days' test is not too much for a rat to take at once, it may be given as one dose at the beginning of the curative period (Coward and Key, 1934). In testing a substance such as butter, of which 0·5g. is a convenient daily dose, the whole 10 days' dose, 5·0g. would be too much to give to a rat on one day, and it should be given as daily doses. According to Coward and Key, the whole dose of Standard may still be given as one dose at the beginning of the curative period, but according to Bacharach (1936), the dose of Standard should be divided into daily doses also, for he found in his colony that a given amount of vitamin D was more effective when given in daily doses than when given as one dose.

A micrometer syringe is useful for giving small doses of cod liver oil or of the Standard, accurately. One worker should hold the rat firmly with its mouth open, and the end of the syringe well within the rat's mouth, while another delivers the required dose from the syringe. The rats should be weighed twice during the test period. The result from any rat which has lost 3–4g. during the time should be regarded with suspicion for spontaneous healing may take place during loss of weight.

The amount of food eaten by each rat per day should be recorded. Ten grams of food may be weighed into the food pot the first day and the part not eaten weighed the next day. This is made up to 10g. (or more if required) and the process repeated daily throughout the test. Loss of weight may be accompanied by spontaneous healing of rickets.

(iii) *Number of rats in a test.*—If a curve of response relating healing to dose of vitamin D given has been constructed and information concerning the probable potency of the test substance is available, then 8–10 rats should be used for the test substance and an equal number for the Standard. Each litter used should be divided equally between test substance and Standard, and within each litter, animals should be paired by

initial weight as far as possible. The sex of the animals seems to be immaterial according to Coward and Key (1933), and according to Bourdillon, Bruce, Fischmann and Webster (1931), but not according to Bacharach (1936). All the rats allocated to the test substance should be given the same dose, and those allocated to the Standard the same dose also. A useful dose of the Standard is 0·5 unit daily or 5 units as one dose, and of cod liver oil, 5mg. daily or 50mg. as one dose. These doses should give lines of healing, under the conditions of the test, wide enough and yet not too wide to allow a fair range in differences in width to be measured.

If no curve of response has been constructed, then 6 litters of 6 rats each should be used, 1 rat from each litter being given one of the doses, 0·25, 0·5, 1·0 unit daily (or 2·5, 5·0, 10·0 units as one dose) or 2·5, 5·0, 10·0mg. cod liver oil daily (or 25, 50, 100mg. as one dose). Thus there would be 6 rats on each of the six doses, 36 in all. This arrangement of the test is usually made when no information of the probable potency of the substance is available. When such information is available, then the number of groups may be reduced to three, two of these being given doses of the Standard (say, 5 units or 10 units as one dose to each rat) and the other group being given a dose of cod liver oil which is expected to contain between 5 and 10 units. It would be desirable then to use 8–10 animals in each group.

(iv) *Measurement of healing by the macroscopic examination of the cut bones.*—At the end of the 10 days' curative period the rats are killed by coal gas or ether. The forelegs are cut off about the middle of the radius and ulna, the skin drawn back over the paws and the distal ends of the radius and ulna removed. A label bearing the rat's number written in pencil is tied to the two bones and the whole placed in 4% formaldehyde (10% formalin) for a few hours (or days if more convenient) to clear the tissues somewhat. The two bones are then separated by a cut with a sharp scalpel and each bone cut longitudinally in a plane parallel to the plane of the cut made to separate the two bones. The four halves are placed in 1·5% silver nitrate for a few minutes, the cut surface uppermost. They are then placed in distilled water and exposed to light. The phosphates of the bone are changed to silver phosphate which is converted into colloidal (black) silver on exposure to

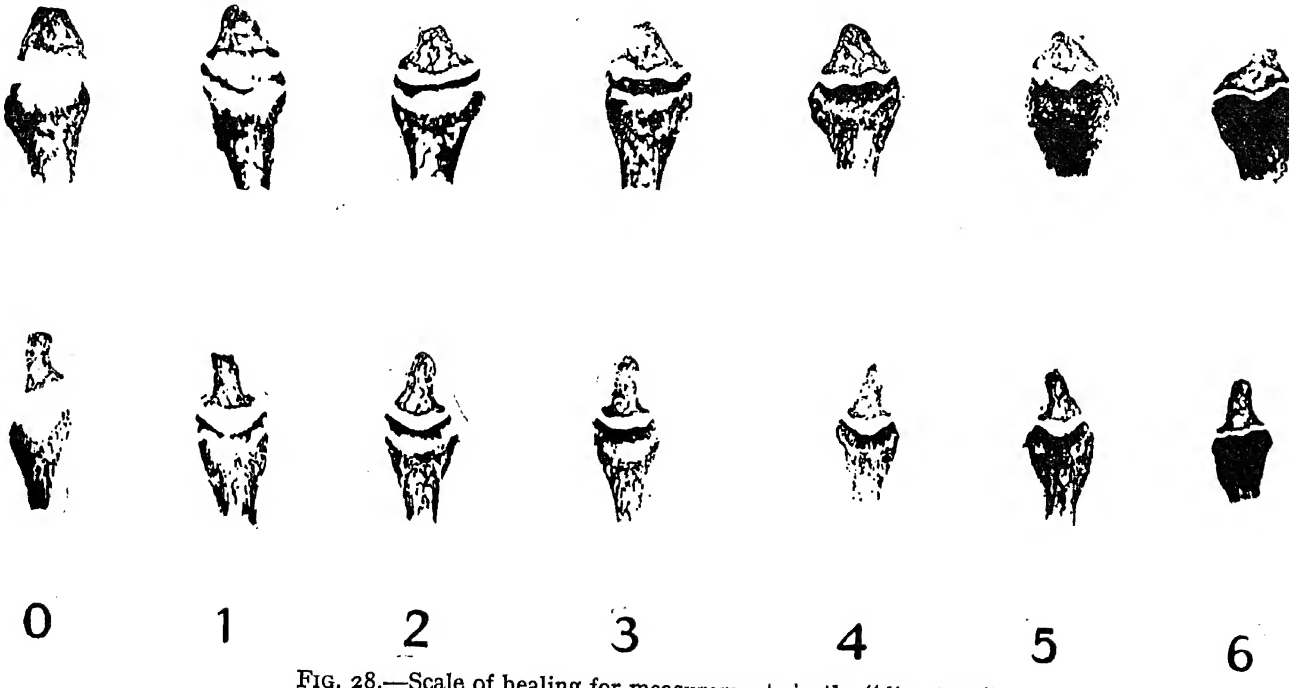


FIG. 28.—Scale of healing for measurements in the "line test."

light. Thus the metaphysis which consists of cartilage and is not stained is easily recognised and the blackened "line" in it (if any) can be measured and a value assigned to it. The healing of the rats given a dose of test substance and a dose of Standard respectively are averaged.

To average the healing of different bones, each one must be assessed by comparison with a scale of a series of bones showing graded amounts of healing to which numerical values have been given. A scale of this kind has been drawn up in the laboratories of the Pharmaceutical Society of Great Britain,

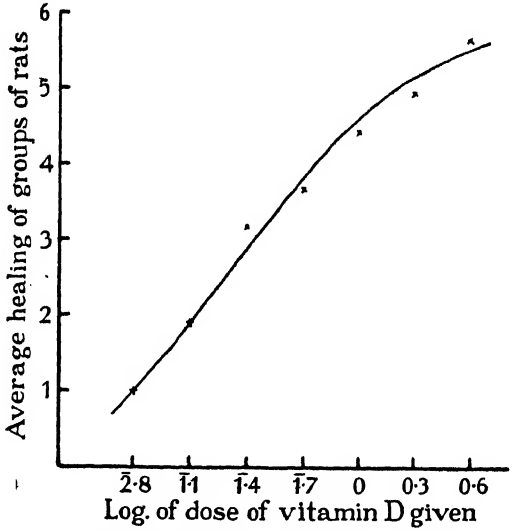


FIG. 29.—Curve of response relating average healing of groups of rats to the logarithm (to the base 10) of the dose of vitamin D given.

and has been in use there for several years (Fig. 28). Six stages of healing are represented, each stage from 1 to 4·5 being produced by approximately double of the dose required to produce the stage below it on the scale. This was determined after the scale had been drawn up and a curve of response constructed by the method described in an earlier part of this chapter. The curve may be seen in Figs. 25 and 29. It is obviously logarithmic throughout the greater part of its length. Different workers do not always assess the amount of healing in a particular bone equally. It has happened that one worker in a laboratory almost consistently assesses a bone half a degree

higher than another worker, but at the range at which this test is most accurate this affects the result very little. When the assessments vary by half a degree either way the averages from 10 rats generally agree so well that the results obtained by two workers do not differ by more than 5%.

The healing can, however, be measured mechanically, with possibly more accuracy. Morgan (1932) made camera lucida drawings of his " line test " bones with constant magnification and then measured the area of new calcification by means of a planimeter. When a less densely calcified part was distinguishable from the very densely calcified part he assigned only half the value to the less dense part.

(v) *Working out the result.*—(*a*) By the use of a curve of response previously constructed. When the results from the rats given the doses of test substance and Standard respectively have been averaged, the averages are compared by means of the curve of response. The abscissæ corresponding to the average results are determined from the curve. Then the ratio of the potencies of the two doses is the ratio of these abscissæ and the potency of the test substance is calculated.

Example.—The following is a result obtained recently in the writer's laboratory :

Dose.	Litter.	Rat.	Healing.	Average healing.
25mg. cod liver oil	3393	9277	3·5	2·44
		9279	2·0	
		9280	1·5	
		9282	2·0	
	3399	9312	3·5	
		9313	2·0	
	3403	9338	2·0	
		9342	3·0	
2·5 units vitamin D International Standard.	3393	9278	2·5	2·06
		9281	1·5	
		9283	2·5	
		9284	1·5	
	3399	9315	2·0	
		9316	3·5	
	3403	9340	1·5	
		9341	1·5	

The abscissa corresponding to the average healing of 2·44 on the curve of response relating healing to dose of vitamin D given in this laboratory is 0·184.

The abscissa corresponding to the average healing of 2·06 on the same curve of response is 0·142.

Thus the ratio $\frac{2{\cdot}44}{2{\cdot}06}$ for healing corresponds to the ratio $\frac{0{\cdot}184}{0{\cdot}142}$ for dose of vitamin D given. Therefore 25mg. cod liver oil contain $\frac{0{\cdot}184}{0{\cdot}142} \times 2{\cdot}5 = 3{\cdot}24$ units of vitamin D. The oil contains 130 International units of vitamin D per gram.

(*b*) Without the use of a previously constructed curve of response.

If three groups only have been used for the test, two groups having been given doses of the Standard (say, 5·0 and 10·0 units respectively as one dose to each rat) and one group having been given a dose of cod liver oil (say, 50mg. to each rat) then the results from the rats in each group are averaged. Those from the two doses of the Standard are plotted against the logarithms of the doses and the points so obtained are joined by a straight line. This procedure assumes that the relation between effect and dose is logarithmic. This has, in fact, been shown to be so by many workers. The abscissa corresponding to the average result from the cod liver oil is found, and since the abscissa is the log. of the dose, the dose is found by determining the antilog. of the abscissa. This then is the number of units of vitamin D in the dose of cod liver oil given. The potency is stated as the number of International units per gram of oil.

Example.—Suppose two groups of rats, given 5 units and 10 units respectively of vitamin D per rat as one dose, showed average healing of 1·40 and 2·19 scale divisions. The results 1·40 and 2·19 are plotted as ordinates against the logs. of 5 and 10, *i.e.* against 0·6990 and 1·0, as abscissæ. A straight line is drawn between the two points so obtained (Fig. 30). Suppose the average healing of the rats given 50mg. cod liver oil was 1·61. The abscissa corresponding to this on the curve is 0·78. The antilog. of 0·78 is 6·026. Therefore 50mg. cod liver oil contain 6·026 units of vitamin D and the cod liver oil contains 120 units of vitamin D per gram. It would be absurd to state the potency more accurately than this.

If three groups of rats are given different doses of the

Standard, then the points obtained by plotting the three average results against the logs. of their respective doses are joined by the best straight line whose position is found by the method described in Chapter III. If three other groups of rats have been given different doses of cod liver oil, or any other test substance, the result for each group is worked out as described above and the three determinations averaged.

The straight line drawn between the points determined from the results from the doses of Standard may be prolonged a little way in either direction for the interpretation of results from the test substance lying outside the results from the

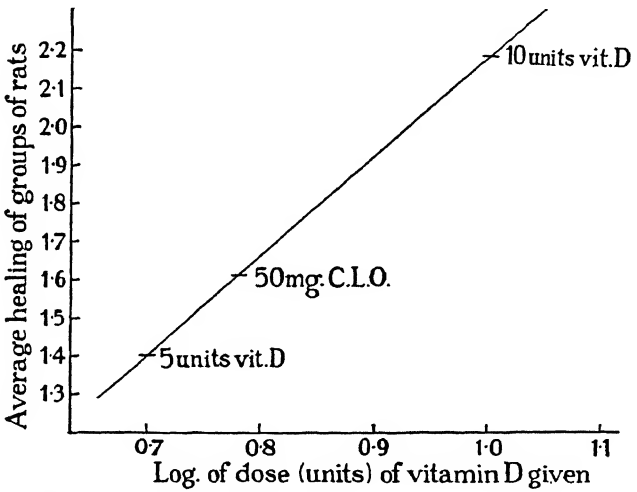


FIG. 30.—Method of working out a result when no previously constructed curve of response is available and three groups of rats have been given two doses of vitamin D and one dose of cod liver oil respectively.

Standard. But extrapolation is always somewhat risky and it should not be carried far.

Both of these last results may be calculated without actually drawing the curve of response. In the first one, the general curve of response constructed previously had been found to show an additional degree or scale division of healing for each successive doubling of the dose. Therefore a difference of one scale division between the average healings of two groups of rats corresponds with a difference of 0·30103 (log. 2) in the logs. of the doses of vitamin D given. Hence a difference of 0·38 (2·44—2·06) of a scale division corresponds to a difference of

$\frac{0{\cdot}30103 \times 0{\cdot}38}{1} = 0{\cdot}1144$ in the logs. of the doses given. Therefore 25mg. cod liver oil which produced an average healing of 2·44 is 1·301 (antilog. 0·1144) times as potent as 2·5 units of vitamin D which brought about an average healing of 2·06. Thus 25mg. cod liver oil contain $1{\cdot}3 \times 2{\cdot}5$ units $= 3{\cdot}25$ units of vitamin D and the oil contains 130 International units of vitamin D per gram.

In the second example, a difference of 0·79 scale division (2·19—1·40) corresponded to a doubling of the dose of Standard, *i.e.* 0·79 scale division corresponded to a difference of 0·30103 in the logs. of the doses of vitamin D given. Hence a difference of 0·21 scale division (1·61—1·40) corresponded to a difference of $\frac{0{\cdot}30103 \times 0{\cdot}21}{0{\cdot}79} = 0{\cdot}0800$ in the logs. of the doses given. Therefore 50mg. cod liver oil which produced an average healing of 1·61 is 1·202 (antilog. 0·0800) times as potent as 5 units of vitamin D which brought about an average healing of 1·40. Thus 50mg. cod liver oil contain $1{\cdot}202 \times 5$ units $= 6$ units of vitamin D, and the oil contains 120 International units of vitamin D per gram.

B. **The "X-ray" method, generally used as a curative test.**

(i) *Preparatory and curative periods.*—Bourdillon, Bruce, Fischmann and Webster (1931) worked out the details of this method which they used extensively in their work on the preparation of calciferol from irradiated ergosterol. They found that a preparatory period of 14 days' feeding on Steenbock's rachitogenic diet, No. 2965, was suitable for rats from their colony. Their curative period was 14 days also. The other details of the procedure for carrying out the determination were similar to those described for the line test.

(ii) *Measurement of healing.*—The bones that were examined by X-ray photography were the proximal ends of the tibiæ. Each rat was photographed twice, once under anæsthesia before dosing and once when killed after 14 days of dosing. The scale of healing used for assessing the healing of the experimental rats was divided into 12 divisions. The procedure for photographing the rats' bones is here quoted at length from the Report on the Quantitative Estimation of Vitamin D by

Radiography, by Bourdillon, Bruce, Fischmann and Webster (1931).

"*Apparatus.*—The X-ray plant consisted of a dental-type, hot kathode tube supplied with 4 amps-filament current, and about 12m.a. high tension current at about 35,000 volts, from transformers with suitable regulating resistances. The voltage was kept as constant as practicable, and an exposure of exactly 5 seconds was ensured by an automatic shutter which was kindly constructed for this purpose by Dr. E. Schuster. The distance from the focal spot on the kathode to the plate was 16".

"The rats were held in a carrier which retained one foot by a light spring clip, in such a position that the knee-joint was kept extended. This carrier allowed for the photography of 10 legs on one 10×4" plate. All plates were developed for a constant time, and at a constant temperature, as uniform exposure and development is of importance in order to facilitate accurate evaluation of calcium deposits. Our photographs appear nearly uniform in contrast (except the earlier series taken with a 'gas' X-ray tube), but sometimes vary perceptibly in density, owing, we believe, to slight variations in the temperature of the developer. However, such variation did not occur between the photographs of any one plate, and as only one plate was used for each litter it could not appreciably influence results.

"*Anæsthesia.*—Before the first radiograph the animals were anæsthetised by being placed on a shelf in a 2-litre glass desiccator containing ethyl chloride vapour. The ethyl chloride was carefully measured before vaporisation, the amount used being 1·5cc. for the first animal in a series, and 0·4cc. or less for each subsequent animal.

"The depth of anæsthesia was judged by observing the rate of loss of consciousness, and the changes in respiration. The average time of exposure to the vapour was 15–30 seconds. The animals appeared to recover completely almost immediately after the radiography.

"It was found that one operator could anæsthetise and radiograph 35–40 live rats per hour, and could radiograph dead rats at a much faster rate."

The X-ray photograph gives a picture of the healing bone very similar to that given by the line test, except, of course, that the line test shows only the section of the layer of healing

and is, therefore, sharper in outline than the X-ray picture which shows the whole thickness of the layer of healing. Each method obviously needs experience in reading the healing.

Assessment of healing from the X-ray photographs is made by comparison with a scale of bones showing graded stages of healing. Bourdillon's scale, reproduced in Fig. 31, consists of 12 stages. He found that doubling the dose of vitamin D resulted in additional two-scale divisions of healing. This is comparable with the finding of Coward and Key (1933), since Bourdillon has twelve divisions in his scale of healing and Coward has six. It indicates also that the rats of the two colonies must have been reduced to rachitic states of similar severity, although the treatment of the two was different.

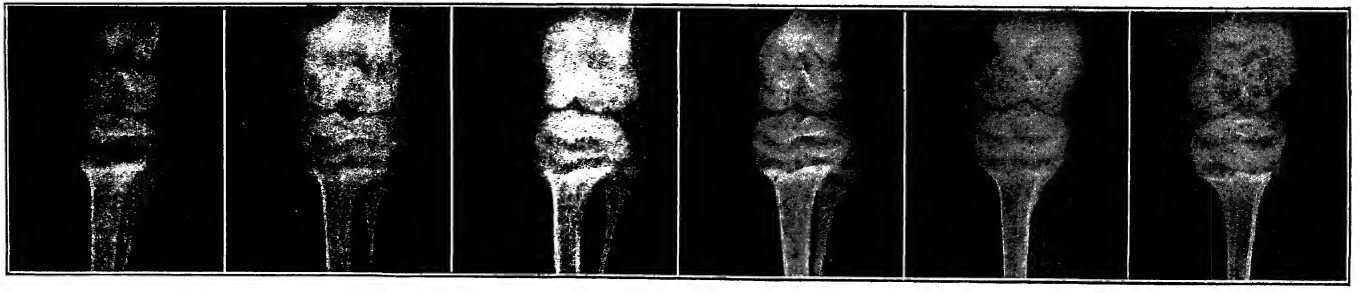
(iii) *Working out the result.*—Bourdillon's method of working out results is similar to the one used for working out the results obtained by the line test. If one member of a pair of rats from the same litter has been given a dose of cod liver oil and the other member of the pair a dose of the Standard, the difference in healing (as scale divisions) between the 2 rats is determined. Then, since a difference of two scale divisions corresponds to a doubling of the dose of vitamin D, the difference in healing is proportional to the difference between the logs. of the doses of vitamin D given. A simple proportion sum and converting the answer into its antilog. gives the ratio of the vitamin D content of the doses given to the 2 rats. Another proportion sum and the averaging of results from, say, 10 such pairs of animals, gives the potency of the cod liver oil.

Example.—Suppose one member of a pair of rats had been given 5·0mg. cod liver oil daily for 14 days, and the other member had been given 0·5 unit vitamin D daily for 14 days. The healing of the first rat was found to be seven scale divisions and the healing of the other rat was found to be six scale divisions.

The difference between the healing of the 2 rats was one division. Then,

since two scale divisions correspond to 0·30103 (log. 2)
one scale division corresponds to 0·15051 which is the log. of 1·415.

Therefore 5·0mg. cod liver oil which produced healing of seven scale divisions contains 1·415 times as much vitamin D as the



1 2 3 4 5 6

7 8 9 10 11 12

FIG. 31.—Scale of healing in rachitic rats enlarged nearly 2 diameters.

[*To face page* 120.

dose of 0·5 unit which produced healing of six scale divisions *i.e.*

$$\frac{5\cdot0\text{mg. cod liver oil}}{0\cdot5\text{ unit vitamin D}}=1\cdot415$$

Therefore 5·0mg. cod liver oil contain

$$1\cdot415\times0\cdot5\text{ unit}=0\cdot7075\text{ unit}$$

The average of ten such results would give a very fair estimation of the potency of the cod liver oil.

It must be clearly recognised that anyone else adopting the X-ray method of determination of vitamin D would have to construct his own curve of response, *i.e.* determine the relation of effect to dose given. He could not apply Bourdillon's curve to his own results, though he could use Bourdillon's scale of healing for assessing the healing of his rats.

C. **The " bone-ash " method, generally used as a prophylactic test.**

(i) *The test period.*—In this method of determination of vitamin D, the doses of test substance and of Standard are given from the beginning of the experiment, doses being chosen of about one-fifth the amount of the doses suitable for a curative test. Doses may be given once or twice a week instead of daily. Each rat should be kept in a separate cage throughout the experiment. Either of the rachitogenic diets already described is suitable for this type of test.

(ii) *Number of rats in a test.*—About six litters of 7 rats each are divided into seven groups, 1 rat of each litter in each group. The rats of one group are given no dose of test substance or of Standard, the rats of the other groups being given doses of test substance in the ratio 4 : 2 : 1 or of Standard in a similar ratio, every rat of any one group being given the same dose. This is continued for 4 or 5 or 6 weeks, the longer the experiment the greater being the " spread " of the results obtained at the end.

If there is no information available as to the possible potency of the test substance a preliminary test must be made with doses chosen over a much wider range than the one proposed, say, in the ratio 9 : 3 : 1 or even wider still, the doses of the Standard being two (ratio 3 : 1 or 2 : 1) known to be suitable to this type of experiment.

(iii) *Measurement of calcification.*—The percentage of ash in the fat-extracted dry bone is required in this method of determination. The femora (or the humeri) of the rats are removed and freed as completely as possible from adhering tissue. Rubbing with cheese cloth is helpful in this. Each bone is broken in two, tied up in a piece of muslin and extracted for at least 6 hours with alcohol in a Soxhlet extractor. The time in the Soxhlet can be shortened if desired by previous boiling in a large volume of alcohol. The bones are then dried to constant weight. Each bone is ashed, by heating in a crucible first over a Bunsen burner, then in a muffle furnace, to constant weight, and the ash content thus determined. The result is expressed as the percentage of ash in the dry, extracted bone. The results from the animals in each group are averaged and the averages compared. If the doses have been suitably chosen one experiment with 42 rats will give a very fair estimate of the potency of the test substance; if not, a second experiment will be necessary with doses of more nearly equal potency.

Example.—A particular sample of cod liver oil was examined by this method. The results are collected in Table IX. The potency of the oil may be determined from the following considerations:

(*a*) 0·8mg. cod liver oil contains more than 0·1 International unit of vitamin D; therefore 1mg. contains more than 0·125 unit.

(*b*) 0·4mg. cod liver oil contains more than 0·05 International unit of vitamin D, but less than 0·1 unit; therefore 1mg. cod liver oil contains more than 0·125 and less than 0·25 unit.

(*c*) 0·2mg. cod liver oil contains more than 0·025 International unit of vitamin D, but less than 0·05; therefore 1mg. cod liver oil contains more than 0·125 and less than 0·25 unit.

(*d*) 0·2mg. cod liver oil contains more nearly 0·05 unit than 0·025 unit; therefore 1mg. cod liver oil contains more nearly 0·25 unit than 0·125 unit.

Therefore the vitamin D potency of the oil may be estimated at about 200 International units per gram.

A more accurate way of dealing with results such as these is to plot each series of results against the logarithms, to the base 10, of the doses given (mg. cod liver oil or mg. International Standard) on the same graph, draw the best straight line through

TABLE IX

THE DETERMINATION OF THE VITAMIN D CONTENT OF A SAMPLE OF COD LIVER OIL BY THE " ASH CONTENT OF THE BONES " METHOD

Cod liver oil.						International Standard.					
Daily dose.	Rat.	Weight of dry extracted bone.	Weight of ash.	Percentage ash in dry extracted bone.	Average percentage ash.	Daily dose.	Rat.	Weight of dry extracted bone.	Weight of ash.	Percentage ash in dry extracted bone.	Average percentage ash.
0·8mg.	♀ 185	0·1172	0·0504	43·00	45·86	0·1 unit	♀ 186	0·1211	0·0430	35·51	40·79
	♀ 238	0·1249	0·0575	46·04			♀ 240	0·1057	0·0437	41·34	
	♂ 294	0·1606	0·0759	47·26			♂ 295	0·1345	0·0563	41·86	
	♂♀ 347	0·1427	0·0660	46·25			♂ 348	0·1227	0·0525	42·79	
	♀ 256	0·1230	0·0600	48·75			♀ 258	0·1119	0·0472	42·18	
	♂ 368	0·1369	0·0600	43·83			♂ 371	0·1244	0·0511	41·08	
0·4mg.	♀ 182	0·0976	0·0344	35·25	36·52	0·05 unit	♀ 183	0·0830	0·0312	37·59	32·52
	♂ 235	0·1069	0·0389	36·39			♀ 239	0·0945	0·0288	30·48	
	♂♀ 296	0·1317	0·0476	36·14			♂ 297	0·1228	0·0404	32·90	
	♀ 351	0·1098	0·0416	37·89			♂ 346	0·0848	0·0282	32·25	
	♀ 257	0·0815	0·0291	35·71			♂♀ 259	0·0914	0·0270	29·54	
	♂ 369	0·1089	0·0411	37·74			♂ 370	0·0878	0·0284	32·36	
0·2mg.	♂ 181	0·1000	0·0249	24·90	30·16	0·025 unit	♀ 184	0·0780	0·0161	20·64	23·38
	♂♀ 237	0·0909	0·0293	32·23			♂♀ 236	0·0883	0·0222	25·14	
	♀ 298	0·0961	0·0324	33·71			♀ 299	0·0992	0·0238	23·99	
	♀ 350	0·0894	0·0281	31·43			♀ 349	0·0829	0·0191	23·04	
	♂ 254	0·1080	0·0265	24·54			♂ 255	0·0842	0·0208	24·70	
	♀ 373	0·0922	0·0315	34·16			♀ 372	0·0804	0·0183	22·76	

the two series (either by eye or mathematically as described on p. 21) and then, if the two lines are nearly parallel, as they are in this experiment, find the abscissæ of each curve corresponding to one particular percentage of ash and equate the doses. In Fig. 32 curve A is the curve of response to doses of the Standard in mg., curve B is the curve of response to doses of the cod liver oil in mg.

30% ash corresponds to abscissa $\bar{2}{\cdot}625$ on the curve for the Standard,

30% ash corresponds to abscissa $\bar{1}{\cdot}315$ on the curve for this cod liver oil.

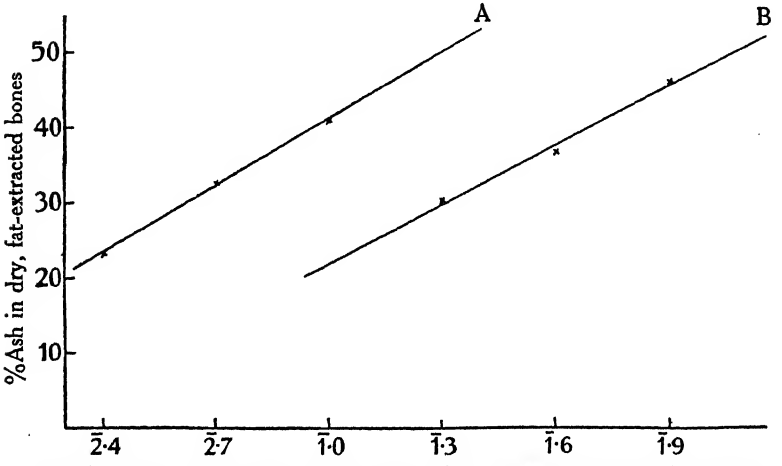


FIG. 32.—To determine the potency of a sample of cod liver oil by the "ash-content of the bone" method. Three groups of rats have been given doses of 0·2, 0·4, 0·8mg., respectively, of cod liver oil, and three other groups doses of 0·025, 0·05 and 0·1mg., respectively, of the International Standard for vitamin D.

Then the antilog. of $\bar{2}{\cdot}625$ as a dose of the Standard in mg. (or unit) corresponds to the antilog. of $\bar{1}{\cdot}315$ as a dose of cod liver oil in mg.; *i.e.* 0·04217 unit is equivalent to 0·20654mg. cod liver oil. Therefore the potency of the cod liver oil is 204 International units of vitamin D per gram. It would be unwise to call this anything but 200 units per gram.

D. The "increase in weight" method.

One attempt has been made to use the increase in weight of the whole animal as the criterion for the determination of

vitamin D (Coward, Key and Morgan, 1932). The basal diet used in the investigation was made as nearly like the one used in the determination of vitamin A as possible, the difference being an absence of vitamin D in the vitamin D test instead of an absence of vitamin A as in the vitamin A test. In particular the same salt mixture was used in both tests; thus there was no disturbing high-calcium, low-phosphorus content for the vitamin D to rectify. The diet consisted of:

Caseinogen, light white (B.D.H.)	15%
Dextrinised rice starch .. .	73%
Dried brewer's yeast .. .	8%
Salt mixture (Steenbock's 40) .	4%

In addition, each rat was given a dose of carotene equivalent to 0·04mg. in 0·02g. olive oil daily, three times a week throughout the whole of the experiment. The composition of the salt mixture was important in this experiment. It consisted of:

Sodium chloride (NaCl)	23·36 parts
Magnesium sulphate ($MgSO_4.7H_2O$) ..	24·6
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	35·8
Dipotassium hydrogen phosphate (K_2HPO_4)	69·6
Calcium phosphate ($CaHPO_4.4H_2O$) ..	68·8
Calcium lactate. $5H_2O$	15·4
Iron citrate. $6H_2O$	5·98
Potassium iodide (KI)	0·16

The experiment was carried out like the corresponding experiment with vitamin A. Rats were given this diet until they ceased to put on weight. During this time the members of any one litter were kept together in one cage. They were weighed twice a week, and as each one became steady in weight it was transferred to a separate cage for the rest of the experiment.

(i) *Behaviour of the rats while they were given the basal diet only.*—The increase in weight of the rats during the time that they were given the basal diet only varied greatly. Some increased only a few grams in weight, others increased as much as 60–70g., and a few had to be discarded for they attained too high a weight. Those that grew did so at a slow rate, much slower than rats given a vitamin A-free diet, and when they ceased to gain in weight, and were given no dose of vitamin D, they remained stationary for many weeks before dying, and sometimes died without much loss in weight. This again was very different from the behaviour of animals

given a vitamin A-free diet, which lose weight rapidly after once becoming stationary in weight. This suggests that the need of the rat for vitamin D is less than its need for vitamin A.

(ii) *Response to doses of vitamin D.*—Daily doses of 0·01, 0·05, 0·1, 0·2, 0·25, 0·4, 0·75, 0·8 unit in olive oil were tested on groups of rats which had become steady in weight on the basal diet. Two of these doses (0·25 and 0·75) are unnecessary. For a fuller account of the experiment see Chapter VII. The groups consisted of much smaller numbers of rats than had been used in constructing the curve of response to vitamin A in the same laboratory, but the average responses showed clearly that there was a graded response to graded doses of vitamin D.

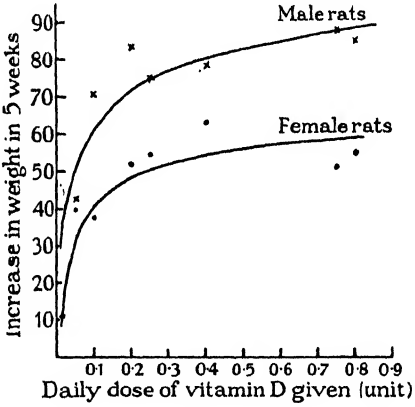


FIG. 33.—Curves of response of male and female rats relating increase in weight in 5 weeks to the dose of vitamin D given.

The curvilinear relationship was evident even when the results were separated into those from male and female rats respectively, *i.e.* into groups consisting of much smaller numbers of animals. Both curves of response were logarithmic (Fig. 33).

(iii) *Test of the curve of response.*—The validity of this curve was tested by using this method to examine the potency of a sample of irradiated ergosterol whose potency was determined by the line test as 2,000,000 units per gram.

The test was carried out at the same time that the curve was being constructed. Doses of 0·00001 and 0·0005mg. respectively were tested, the high dose being begun before the

very high potency of the solution was known from the line test. Thus the ratio of the two doses was 50 : 1. The doses of the Standard which would have brought about the same increases in weight as the doses (0·00001 and 0·0005mg. solution respectively) were found from the curves of response and the potency of the solution thus determined. The figure actually found was 2,068,500 units per gram, which, by its confirmation of the potency determined by other workers using the line test, indicated that the increase in weight of the rats had determined the same substance that had been determined by the other method.

An investigation of the accuracy of this method proved to be disappointing, however. It was much less than that of the determination of vitamin A by a similar method. For details of this calculation see Part II, Chapter XII.

E. Comparison of the four methods.

The best criterion to use for determining the vitamin D content of a substance is the ash content of the bones. This measures the whole calcification of a bone or bones which may be considered representative of the whole skeleton. It is the most accurate of the methods in general use (see Chapter XII). It has, however, the disadvantage of being very laborious and time-consuming. It requires at least 4, and preferably 5 or 6, weeks of feeding the animals with special doses daily or twice weekly, and the determination of the ash content of the bones after this adds greatly to the labour involved.

The " line test " and " X-ray " method involve about the same amount of work both in feeding the animals and in examining the bones afterwards. Both are more easily carried out than the " ash content of the bones " method for they involve shorter periods of special feeding and shorter treatment of the bones. They are, however, less accurate and subject to the personal error involved in assigning a value to each bone from a comparison with a series of bones showing a scale of healing. Both can be recommended for routine testing, the " X-ray method " having one disadvantage over the " line test " in the cost of the apparatus needed for taking the X-ray photographs.

The " increase in weight " method has little to recommend it. The stage at which the rat is ready for doses is ill-defined,

the period of feeding is long (at least 3 weeks) and the accuracy of the test is low. The criterion is not even specific for vitamin D. Its one advantage lies in the fact that the measurement of increase in weight is independent of any personal judgment.

4. The Difference in Results obtained by using (a) Rats and (b) Chickens, in the Determination of Vitamin D

Massengale and Nussmeier (1930) first showed that cod liver oil was much more effective in promoting calcification of bones of chickens than was irradiated ergosterol when the two were given in doses equivalent in potency according to previous determinations on rats. This has since been confirmed by other workers. It has led to the conclusion that the vitamin D of cod liver oil is not the same substance as the vitamin D of irradiated ergosterol and that the chicken cannot make use of the vitamin D of irradiated ergosterol to anything like the same extent as it can make use of the "natural" vitamin D of cod liver oil. This means that if a sample of cod liver oil were compared with the International Standard by testing on chickens it would be found to have a very much higher vitamin D value than if the comparison were made on rats. Bills, Massengale and Imboden (1934) have even suggested that there are at least two "natural" vitamins D and that they occur in different proportions in different fish liver oils.

This raises many questions concerning the method of determination of vitamin D. In particular, how does the human subject react to the two (or more) forms of vitamin D? Hess thought that human babies made slightly less good use of the vitamin D of irradiated ergosterol than of the natural vitamin D, but an extensive investigation made by Eliot, Nelson, Barnes, Browne and Jenss (1936) on 600 children showed that they used the two forms of vitamin D equally well; that is, in this respect the human subject behaves like the rat and not like the chicken.

This point has great practical importance. A preparation of calciferol, the vitamin D of irradiated ergosterol, which is intended for clinical work on children, may be assayed by comparison with the International Standard by tests on rats.

It is obvious that it is useless to add calciferol to cod liver oil which is intended for chickens. The potency would be scarcely any greater than that of the unfortified oil. It follows that when a sample of cod liver oil has been assayed by experiment on rats (the only method recognised at present by the Permanent Commission on Biological Standardisation of the League of Nations) the poultry dealer must demand a guarantee from the vendor of the cod liver oil that it is, in fact, a natural cod liver oil without any added calciferol.

There is a desire on the part of some poultry dealers that the cod liver oil that they buy shall have been assayed on chickens. Obviously such an assay cannot be carried out by comparison with the International Standard. It is possible, however, to use the U.S.P. reference cod liver oil as a standard of comparison. Its potency as determined by tests on rats has been estimated as 95 International units per gram. It may be provisionally regarded as containing 95 units of vitamin D per gram for chickens also, but these must not be called International units for there is no accepted International unit other than the unit as determined by tests carried out on rats. It is indeed highly desirable that no fresh unit should be used until there is an actual standard of reference for chicken tests which will be, ideally, the pure form of vitamin D which the chicken uses and of which a certain weight shall be accepted as containing 1 unit. According to the work of Waddell (1934) and Grab (1936) one form of vitamin D which the chicken can use should soon be available in a pure, or nearly pure, form.

With such a standard of reference, assays on chickens can be arranged in the same way as assays on rats. Half of the chickens would be given the Standard, half would be given the cod liver oil. Each substance would be tested on two or three groups, the different groups being given doses of the Standard, or of the cod liver oil, in the ratio 4 : 2 : 1. The potency of the cod liver oil would be determined from the results, as in a determination carried out on rats.

Diet for chickens to be used for determinations of vitamin D.— A diet devised by Hart, Kline and Keenan (1931), and used extensively by them and also by other workers, consists of the following :

59 parts of ground yellow corn.
25 parts of wheat middlings (standard).
12 parts of crude casein.
1 part of common salt.
1 part of precipitated calcium carbonate.
1 part of precipitated calcium phosphate.
1 part of dried yeast.

This ration contains from 19 to 20% of protein, 0·9 to 1·0% of calcium and 0·5 to 0·6% of phosphorus. Hart uses day-old chicks for his experiments and a 5 weeks' prophylactic test.

Criteria for use in determinations of vitamin D by means of chickens.—The curative type of experiment and the "line test" as used on rats cannot be used on chickens, for healing in chickens does not show itself as a line of calcification across the metaphysis in the cut bone; it is diaphyseal and immediately contiguous with the trabeculæ that remain after rickets is produced.

The "ash content of the bone" is the criterion used in determining the calcification produced in chickens by giving vitamin D. The tibiæ are the bones generally used. Their ash content is determined as in rats, but, being rather large bones, it is necessary to crush them somewhat and extract with hot 95% alcohol for a longer period, 72 hours. They are then dried, weighed and ashed in a muffle furnace for 1 hour at about 650° C. The percentage of ash in the dry, extracted bone is calculated.

Lachat (1935) has collected a very large amount of information (by means of a questionnaire) concerning the details of chicken tests as carried out in twenty-three different institutions. He has summarised this in a report which will prove of immense value to workers who are already engaged in this field and to others who contemplate such work.

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CHAPTER VII

THE INTERDEPENDENCE OF THE VITAMINS

It is well known that when young rats are given a diet deficient in vitamin A, they eventually lose weight and die, no matter how much of the other vitamins may have been given to them throughout the experiment. Rats also die if their diet contains abundance of all the vitamins except vitamin B_1. Guinea-pigs die if their diet contains abundance of all the vitamins except vitamin C and rats die in time if their diet contains no vitamin D, even though it contains all the mineral elements in the most satisfactory proportions known. Thus a complete deficiency of any one vitamin cannot be rectified by giving an abundance of the other vitamins.

1. The Influence of a Partial Deficiency of one Vitamin in the Basal Diet on the Determination of another Vitamin

There is a certain amount of evidence in the literature that when the supplies of one vitamin are limited the effect of giving

a small dose of a " test " vitamin is less than the effect would have been if the supplies of the other vitamins had been larger throughout. Consequently a larger dose of the " test " vitamin has to be given in order to bring about a result equal to the result that a small dose of " test " vitamin would have given in the presence of large supplies of the first vitamin. Perhaps the best way to demonstrate this point is to describe the particular experiments which have shown it.

A. **Early evidence of the influence of a partial deficiency of one vitamin on the determination of another vitamin.**

Sherman and Munsell (1925) published a series of results from groups of rats which, having ceased to grow on a diet deficient in vitamin A, were given graded doses, 0, 0·05, 0·1, 0·2, 0·5, 1·0 and 2·0g., respectively, of tomato, every rat of any one group being given the same dose. The experiment was carried on for 8 weeks. It produced a series of composite growth curves whose slopes were nicely graded to the doses of tomato given. Later, Sherman and Batchelder (1931) published another series of composite growth curves of groups of rats, similarly prepared, but given graded doses of dried whole milk (the ratios only of the doses being stated, 0, 1X, 2X, 4X, 8X and 16X). This experiment was carried on for 8 weeks also, and again a nicely graded series of growth curves was obtained ; but it was at once evident that the " spread " of the curves obtained in response to doses of milk was greater than that obtained in response to doses of tomato. Sherman and Batchelder say in their paper : " From the view-point of present-day knowledge, however, it is readily conceivable that the less accentuated response to increasing levels of feeding of the tomato may have been because this was a less adequate source than milk of some factor or factors whose influence upon growth was not fully realised at the time of the earlier experiments or possibly that, notwithstanding the general equivalence of the vitamin A of animal origin and its precursor of vegetable origin, there may still be a difference in the degree of readiness and completeness of utilisation of the two forms which might become measurable in the averages of sufficiently numerous experiments made in as rigorously quantitative a way as our present knowledge and experience permit." It should here be pointed out, however, that in one respect the two experi-

ments differed. In the experiment in which different doses of tomato were given, the basal diet of the rats contained only 5% of dried brewers' yeast, whereas in the experiment in which different doses of dried milk were given the basal diet contained 10% of dried brewers' yeast. This difference may account for the difference in the "spread" of the composite growth curves obtained in the two experiments, for similar differences in the "spread" of results have been obtained by the writer by varying the amounts of one of the vitamin constituents of the basal diet.

B. **The response to doses of vitamin D when the supplies of vitamin A are limited.**

In 1932, Coward, Key and Morgan published an account of an attempt to determine vitamin D by an "increase in weight" method arranged like the "increase in weight" method for the determination of vitamin A.

The basal diet consisted of:

Caseinogen, "light, white" (B.D.H.) ..	15%
Dextrinised rice starch	73%
Dried yeast	8%
Salt mixture (Steenbock's 40)	4%

The salt mixture consisted of:

Sodium chloride, NaCl	23·4g.
Magnesium sulphate, $MgSO_4.7H_2O$	24·6g.
Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$	35·8g.
Dipotassium hydrogen phosphate, K_2HPO_4	69·6g.
Calcium phosphate, $Ca_2H_2(PO_4)_2.4H_2O$	68·8g.
Calcium lactate. $5H_2O$	15·4g.
Iron citrate. $6H_2O$	6·0g.
Potassium iodide, KI	0·2g.

In addition each rat was given a daily dose of 0·04mg. of the light petroleum-soluble fraction of dried carrots as a source of vitamin A. The carrots were minced, spread in a very thin layer on sheets of glass, and dried in a current of air at a temperature of 50–60°. The dried material was then extracted twice with light petroleum and the extract evaporated on a warm sand bath in a current of nitrogen. The oily residue was diluted with olive oil to such a concentration that the required amount was contained in 0·02g. oil which was given daily as one drop directly into each rat's mouth. Fresh supplies of this preparation were made about twice a week. It was not

realised at the time that vitamin A probably exists only as the provitamin, carotene, in plant tissues. The dose of unsaponifiable matter contained only about 0·2γ carotene, which is now known to be much below the daily requirement of the growing rat.

The experiment was carried out as described in Chapter VI. Briefly, the rats were given the basal diet plus the carrot extract until they ceased to grow. As they became steady in weight they were divided into seven groups. The rats of the different groups were given daily doses of 0, 0·01, 0·02, 0·05, 0·1, 0·5 and 1·0 unit of vitamin D, each rat of any one group being given the same dose for 5 weeks. The average increases in weight of the different groups were calculated and plotted against the doses of vitamin D given. A fairly smooth curve of response was obtained. It was, however, decided to determine two more points in the curve from doses of 0·25 and 0·75 unit of vitamin D. Further groups were then formed of rats which had been prepared similarly except that, to economise labour and time, a commercial preparation of carotene (B.D.H.) was used instead of the crude carrot extract prepared in the laboratory. The same weight of carotene as of extract was used for it had been considered that 0·04mg. carrot extract had supplied abundance of vitamin A, and if the same weight of carotene was given that also would supply abundance of vitamin A. The average increases in weight in 5 weeks of these two groups of rats were found, but when applied to the graph of the result from the other groups of rats, they were found not to lie on the curve of response at all, but far above it. As the source and probably the amount of vitamin A was the only factor known to be different in the two experiments, it was decided to test other doses of vitamin D on rats given 0·04mg. carotene instead of carrot extract. The doses then tested were 0·01, 0·05, 0·1, 0·2, 0·4, 0·8 and 10·0 International units, the last being considered a massive dose. Thus altogether nine doses of vitamin D were tested with 0·04gm. carotene as a source of vitamin A. The average increases in weight of the nine groups of rats when plotted against the doses of vitamin D given formed a curve of response which was fairly smooth considering the small number of animals in each group. This curve was decidedly steeper than the one obtained when 0·04mg. carrot extract containing only 0·2γ carotene had

been given as the source of vitamin A (Fig. 34). This was strongly suggestive that the response to one vitamin is influenced by the amount of another vitamin available.

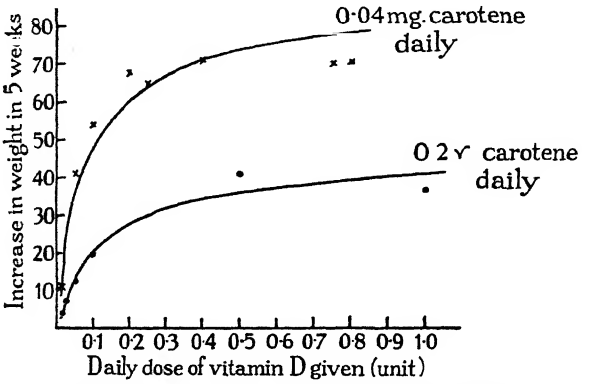

FIG. 34.—The influence of the vitamin A content of the diet on the response to doses of vitamin D.

C. **The response to doses of vitamin B_1 when the supplies of one or more of the other vitamins B (or some other constituent of "autoclaved yeast") are limited.**

Just as Coward, Key and Morgan had found that inadequate supplies of vitamin A in the basal diet modified the response of rats to graded doses of vitamin D, so Coward, Burn, Ling and Morgan (1933) found that inadequate supplies of autoclaved yeast, shown by tests on pigeons to contain no vitamin B_1, modified the response of rats to graded doses of vitamin B_1. In a first attempt to construct a curve of response to graded doses of vitamin B_1 the following basal diet was used:

Caseinogen, light white (B.D.H.)	15%
Dextrinised rice starch	71%
Agar-agar	2%
Salt mixture (Steenbock's 40) (see p. 134)	4%
Dried brewers' yeast autoclaved at 120° for 6 hours, dried at 100° C. overnight and ground finely ..	8%

In addition each rat was given 0·1g. (5 drops) of a good sample of cod liver oil twice a week to supply vitamins A and D.

The brewers' yeast, after autoclaving, did not contain enough vitamin B_1 to cure retracted neck in any of 3 pigeons when a dose of 1·0g. was administered, whereas a dose of 0·03g.

of the International Standard has cured in twenty-one tests from 33 to 86%, and a dose of 0·1g. of different samples of dried yeast (non-autoclaved) has cured from 10 to 87% of the birds used in each test. It was therefore considered to contain negligible quantities of vitamin B_1, if any.

The rats were prepared as described in Chapter IV. Briefly, they were given a preparatory period of 10–14 days when no supplement but cod liver oil was given and they became steady in weight. They were then divided into six groups as nearly comparable as possible regarding weight, sex and litter. Each rat was given a separate cage, with a grid of ½″ mesh. The groups were given daily doses of 0, 0·005, 0·02, 0·04 and 0·1g. respectively of the International Standard for 3 weeks, every rat of any one group being given the same dose.

The average increases in weight of the different groups were calculated and plotted against the dose of Standard given (Fig. 35). A curvilinear relationship was obtained which looked satisfactory until other results obtained at the same time were applied to the curve and it was found that the curve was useless for interpreting these results. Two doses 0·1 and 0·3g. of a certain sample of wheat embryo had been found to produce average increases of weight of −1·12 and +33·37g. respectively in 3 weeks. The abscissa of the curve corresponding to an increase in weight of −1·12g. was found to be 0·008. Therefore the increase in weight produced by giving 0·3g. wheat embryo should have a corresponding abscissa of $3 \times 0{\cdot}008$, *i.e.* 0·024. Actually the increase in weight of 33·37g. from 0·3g. wheat embryo did not fall on the curve at all; the curve had flattened without reaching that point, and the increase in weight corresponding to abscissa 0·024 was 20·25g. Similarly, two doses of 0·05 and 0·2g. dried yeast tested at the same time had given increases in weight in 3 weeks of −2·65 and 42·85g. respectively. The abscissa of the curve corresponding to an increase in weight of −2·65g. was 0·0075. Therefore the increase in weight from 0·2g. dried yeast should have a corresponding abscissa of $4 \times 0{\cdot}0075$, *i.e.* 0·03. Actually the increase in weight of 42·85g. for 0·2g. dried yeast did not fall on the curve at all. Again, two doses of 1·0 and 3·0g. of a certain food preparation also tested at the same time produced increases in weight in 3 weeks of 23·5 and 46·45g. respectively. The abscissa of the curve corresponding to an increase in weight

of 23·5g. was 0·03. Therefore the increase in weight from 3·0g. of the food should have a corresponding abscissa of 3×0·03, *i.e.* 0·09, but again the actual increase in weight was too high to fall on the curve (Fig. 35). It therefore seemed probable that these test substances were supplying some factor necessary for growth which had not been supplied in sufficient amount to the rats used for the construction of the curve, either in their

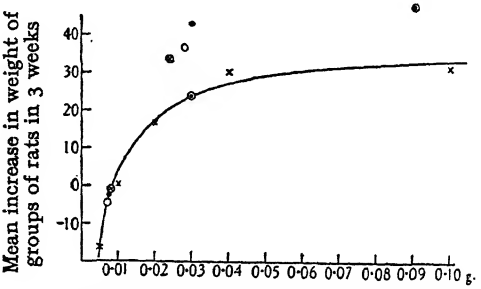

FIG. 35.—A curve of response to doses of the International Standard for vitamin B_1, obtained when only 8% autoclaved yeast was put in the basal diet. It was useless for determining the vitamin B_1 content of other substances, for the higher doses of these other substances produced greater increases in weight than any produced by doses of the Standard.

×=Mean increases in weight of the groups of rats given graded doses of the Standard.
·=Mean increases in weight of groups of rats given daily 0·05 and 0·2g. respectively of a sample of dried yeast I.
⊙=Mean increases in weight of groups of rats given daily 1·0 and 3·0g. respectively of a commercial food sample.
⊗=Mean increases in weight of groups of rats given daily 0·1 and 0·3g. respectively of a sample of wheat embryo.
O=Mean increases in weight of groups of rats given daily 0·05 and 0·2g. respectively of yeast extract I.

The result from the lower dose of each test was plotted on the curve itself. The result from the higher dose of each test was plotted against the abscissa corresponding to the appropriate multiple of the abscissa found for the lower dose.

basal diet or in the different supplements of the International Standard for vitamin B_1. It seemed possible that the autoclaving of the yeast had destroyed not only the vitamin B_1 but also some other factor which is necessary for growth. Chick and Roscoe (1927) showed that when brewers' yeast is heated at 120° C. for 5 hours at its normal *p*H of about 4·5–5·0 it loses half of its vitamin B_2 potency. Williams, Waterman

and Gurin (1929) obtained similar results. It was therefore concluded that although 8% of unheated dried yeast provided ample amounts of all the vitamins B, yet 8% of autoclaved yeast was seriously deficient in vitamin B_2 and possibly in other factors also.

Therefore a new curve of response to doses of 0·005, 0·01, 0·02, 0·04 and 0·1g. International Standard for vitamin B_1

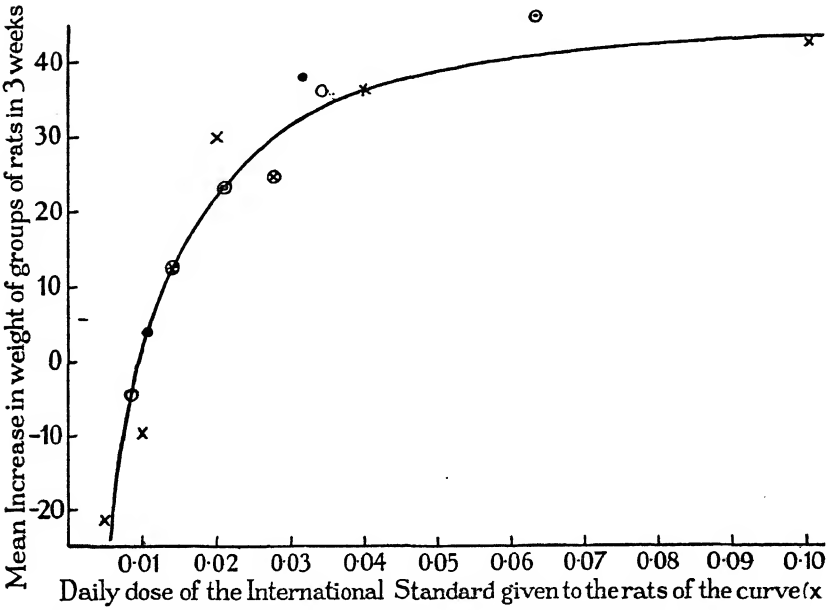

FIG. 36.—A curve of response to doses of the International Standard for vitamin B_1 obtained when 20% autoclaved yeast was put in the basal diet. It could be used for determining the vitamin B_1 potency of other substances, for it was steeper than the one obtained with 8% autoclaved yeast. Presumably 20% A.Y. supplied abundance of the vitamins other than B_1, whereas 8% did not.
Interpretation of symbols as for Fig. 35.

was constructed in the same way as the last, except that the amount of autoclaved yeast (again heated at 120° for 6 hours) was raised from 8 to 20%. The food substances were not retested. The new curve of response (Fig. 36) was much steeper than the former one, and, when the original results from the food substances were applied to it, the actual results were found to be in very good agreement with those calculated by the method described in connection with the first curve (Table X).

As the three substances tested on the curve were of very varied nature (wheat embryo, dried yeast and a more bulky food preparation) and the pairs of doses of each gave concordant results when compared by means of the curve, it was therefore concluded that the second diet provided ample supplies of all growth-promoting factors required for vitamin B_1 tests. Additional support of the validity of the second curve was obtained by testing pairs of doses of three other substances with 20% autoclaved yeast in the basal diet. In all of these the apparent ratio of the potencies of the two doses of a pair, determined from the curve as the ratio of the abscissæ corresponding to the mean increases in weight of the groups of rats used in the tests, was very nearly equal to the known ratio of the doses (Table X).

TABLE X

Substance tested.	Dose, g.	No. of rats.	Mean increase in weight,* g.	Abscissa corresponding to mean increase in weight.	Apparent ratio of doses.	Actual ratio of doses.
Dried yeast I ..	0·05	10	−2·65	0·0096	1 : 4·2	1 : 4
	0·2	10	42·85	0·0407		
Food substance	1·0	6	23·50	0·0226	1 : 2·1	1 : 3
	3·0	6	46·45	0·0467		
Wheat embryo	0·1	6	−1·12	0·0101	1 : 3·5	1 : 3
	0·3	6	33·37	0·0352		
Yeast extract I	0·05	5	−4·60	0·0090	1 : 3·8	1 : 4
	0·2	5	36·15	0·0341		
Dried yeast II	0·05	3	4·2	0·0125	1 : 2·9	1 : 3
	0·15	4	38·0	0·0365		
Yeast extract II	0·1	4	12·8	0·0160	1 : 1·6	1 : 2
	0·2	4	24·5	0·0255		

The difference in slopes of the two curves of response to doses of vitamin B_1 obtained by giving different amounts of

* The numbers of bucks and does in each group were not always equal. Hence, the average results from bucks and does separately were calculated and then the mean of the two averages calculated, so that each average was virtually obtained from an equal number of bucks and does and could therefore be used with the curve which was constructed from equal numbers of bucks and does.

autoclaved yeast in the basal diet demonstrates afresh that the response of animals to doses of one vitamin is influenced by the amount of another vitamin supplied.

D. **The response to doses of vitamin A when the supplies of a certain unknown factor (possibly a vitamin) are limited.**

From time to time Coward and co-workers (1929, 1 and 2, 1930, 1931) have published evidence that a particular kind of caseinogen (light white B.D.H.) possesses greater growth-promoting power than another kind of casein supplied by another commercial firm for purposes of vitamin-testing. Whether the factor is a vitamin or some other factor necessary for growth has never been determined, but certain points in the work suggest that the factor may be a vitamin. Work by Mapson (1932, 1933) suggests that a substance recognised by him in liver and certain liver extracts may actually be the same factor that Coward found associated with caseinogen (and various other substances also, *e.g.* liver, wheat embryo, milk, etc.). In the discussions of papers at the meeting of the American Institute of Nutrition held in Washington, 1936, other workers reported the existence of some substances which may prove to be identical with what has been spoken of in the literature as the " casein " factor (whatever that may be).

This factor is evidently necessary for growth and should therefore be present in abundance in all diets used in " increase in weight " methods. In a paper by Coward, Key, Dyer and Morgan (1930) are described the results of an attempt to construct a curve of response to graded doses of vitamin A when 15% of the " Glaxo " casein was incorporated in the diet as the source of protein. The average increases in weight of the groups of animals given graded doses of a certain sample of cod liver oil were published, but no curve relating mean increase in weight to dose of vitamin A (cod liver oil) given. It is given here, however (Fig. 37), to show graphically what a very shallow and useless curve it was. Recognising the uselessness of such a curve and recognising also that some constituent of the diet must be faulty, Coward, being familiar with Palmer's work on the preparation of different forms of casein, immediately suspected the casein of being somehow inadequate. She then constructed a fresh curve of response to

graded doses of vitamin A, using the same sample of cod liver oil as in the construction of the first curve, and using as a source of protein the caseinogen sold by the British Drug Houses under the name "light white." A very different curve was obtained (Fig. 37); it was much steeper than the first curve and also much smoother. (The greater smoothness of the curve *may* be accounted for by supposing that differences in the different rats' reserves of the "casein factor" were made good by the casein used in the construction of the second curve but not by the casein used in the first.)

Whatever may prove to be the difference between the two

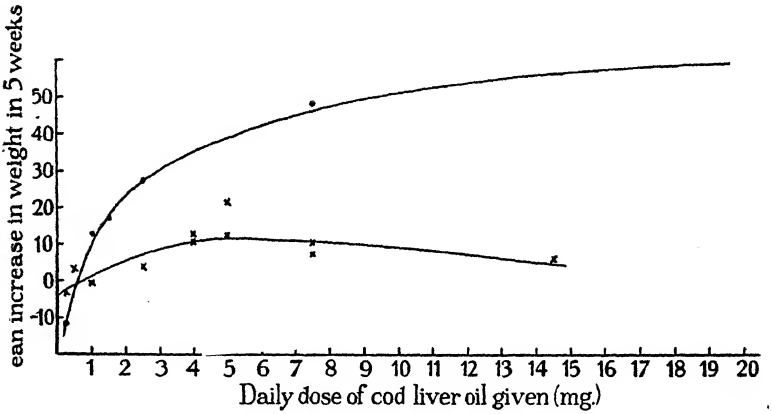

FIG. 37.—Two curves of response relating increase in weight in 5 weeks to doses of the same sample of cod liver oil given, obtained at the same laboratory. The steeper one was constructed when light white (B.D.H.) casein was used as the source of protein, the less steep one when Glaxo "vitamin-free" casein was used.

forms of casein, the fact remains that different curves of response to doses of vitamin A can be obtained by varying one constituent of the basal diet. In order to be sure that a basal diet contains all growth-promoting substances in ample amounts, different substances, as much varied in nature as possible, should be tested, each in two or more doses. The average increases in weight produced by two doses in the ratio of, say, 2 : 1, of any one of these substances should be referred to the curve of response and the abscissæ corresponding to the increases in weight found. If the two abscissæ bear the same (or nearly the same) ratio to each other as the known ratio of

the doses given, then the curve of response may be considered a valid one and the basal diet satisfactory. The greater the ratio between two doses giving concordant results, the stronger is the evidence of the validity of the curve and the adequacy of the diet. Coward put her second curve to this test with the results obtained in Table XI. Considering the varied nature of the substances and the wide range of some of the pairs of doses tested the results indicate that her second curve of response is valid and her basal diet adequate.

It should, however, be emphasised here that a curve of response shown to be satisfactory in one laboratory cannot be assumed to be satisfactory for use in another laboratory. Phillips (1934) has in fact shown that the curves of response to graded doses of vitamin A given by his colony of rats and with the use of his basal diet which is different from Coward's, are represented by the equations :

(bucks) $y^2 = 2{,}992 + 10{,}730 \log_{.10}$ dose (International units)

(does) $y^2 = 1{,}798 + 4{,}350 \log_{.10}$ dose (International units).

Other workers (private communications) have also found their curves of response to graded doses of vitamin A to have different slopes.

2. The Influence of an Excess of one Vitamin in the Basal Diet on the Determination of another Vitamin

After demonstrating the influence of a partial deficiency of one vitamin in the basal diet used on the determination of another vitamin, it seemed necessary to investigate the influence of an excess of one vitamin on the determination of another. An additional reason for making this investigation was the fact that when Culhane and Coward compared two particular samples of cod liver oil they obtained the same ratio for the potencies (3 : 1), yet the average responses of Culhane's groups of rats given certain doses were only about half of the average responses of Coward's groups of rats given the same doses. A comparison of the basal diets used by the two workers showed two main differences. Coward had used light white (B.D.H.) casein whereas Culhane had used a strongly heated casein. Moreover, Coward had given only 8 International units of vitamin D per week to each rat and Culhane had given 200 International units. The first of these differences

was thought to be the probable cause of the differences in response, but the other difference seemed to be worth investigating.

TABLE XI

COMPARISON BETWEEN THE KNOWN RATIO OF PAIRS OF DOSES OF DIFFERENT SUBSTANCES TESTED FOR VITAMIN A AND THE APPARENT RATIO OF THE DOSES AS DETERMINED FROM THE ABSCISSÆ OF THE CURVE OF RESPONSE CORRESPONDING TO THE MEAN INCREASES IN WEIGHT FOUND IN THE TESTS.

When the substance was given as a percentage of the diet records were made daily of the amount of food eaten, and from these were calculated the average amounts of test substance eaten per day throughout the experiment.

Substance.	Dose.	Weighted mean of abscissæ for male and female rats.	Known ratio of doses.	Apparent ratio of doses, *i.e.* ratio of abscissæ.
Dairy butter	0·05g.	1·34	2·0 : 1	2·5 : 1
	0·1g.	3·36		
Vitaminised margarine	0·05g.	1·66	2·0 : 1	2·0 : 1
	0·1g.	3·4		
Cod liver oil C	1mg.	0·41	3·0 : 1	4·3 : 1
	3mg.	1·77		
Cod liver oil G ..	1mg.	0·55	3·0 : 1	2·0 : 1
	3mg.	1·09		
Fish-meal, 2·5% ..	0·23g.	2·37	12·1 : 1	10·8 : 1
25·0% ..	2·79g.	25·6		
Dried milk, 0·4% ..	0·022g.	0·42	7·0 : 1	12·0 : 1
2·0% ..	0·15g.	5·05		
„ 0·2% ..	0·013g.	0·84	12·0 : 1	8·5 : 1
2·0% ..	0·155g.	7·2		
	0·1g.	3·2	8·8 : 1	3·8 : 1
„ 10% ..	0·88g.	12·0		
	0·005g.	0·25	8·0 : 1	7·5 : 1
„ 0·5% ..	0·04g.	1·87		
Vitamin prep., 2%	0·1g.	0·71	3·6 : 1	2·8 : 1
„ 5%	0·36g.	1·99		
„ ..	0·01g.	1·3	2·0 : 1	2·6 : 1
	0·02g.	3·4		
Wheat embryo prep.—				
10%	0·71g.	0·64	4·5 : 2·0 : 1	6·0 : 2·7 : 1
20%	1·56g.	1·74		
40%	3·2g.	3·86		

A. **The influence of an excess of vitamin D on the determination of vitamin A.**

Bruce and Morgan (unpublished results from the writer's laboratory) prepared rats as for a vitamin A determination, giving them the vitamin A-free diet described in Chapter III with the usual supplement of 8 International units of vitamin D per rat per week. As they became steady in weight they were divided into three groups which were given 8, 25 or 100 units respectively of vitamin D per rat per week. All the rats of all three groups were given 2mg. cod liver oil each, per day for 1 week and then 1mg. cod liver oil each, per day, for 4 weeks. The amount was reduced after the first week because it appeared to be a rather high dose. The result may be seen in Table XII.

TABLE XII

TO SHOW THAT EXCESS OF VITAMIN D DOES NOT INFLUENCE THE RESPONSE TO A DOSE OF VITAMIN A. DIFFERENT GROUPS OF RATS WERE GIVEN WEEKLY DOSES OF 8, 25 AND 100 UNITS RESPECTIVELY OF VITAMIN D FOR 5 WEEKS AFTER THEY HAD BECOME STEADY IN WEIGHT ON A VITAMIN A-FREE DIET SUPPLEMENTED BY 8 UNITS OF VITAMIN D PER WEEK. ALL WERE GIVEN A DAILY DOSE OF 2MG. COD LIVER OIL FOR 7 DAYS AND THEN A DAILY DOSE OF 1MG. COD LIVER OIL FOR 28 DAYS.

	No. of rats in group.		Average weight at beginning of prep. period, g.		Average weight when steady, g.		Average weight after dosing with cod liver oil, g.		Increase in weight after 5 weeks, g.	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Rats given 8 International units of vitamin D per week	4	10	27·7	26·0	64·5	72·6	83·5	90·7	19·0	18·1
Rats given 25 International units of vitamin D per week	6	11	28·7	28·4	76·0	72·6	94·0	90·5	18·0	17·8
Rats given 100 International units of vitamin D per week	4	10	27·7	27·7	66·7	69·9	86·7	92·3	20·0	22·4

The differences between the increases in weight of the different groups of rats cannot be regarded as significant. It is evident therefore (*a*) that 8 International units of vitamin D per week were sufficient for rats of this colony, and (*b*) that an excess of vitamin D has no harmful effect on a vitamin A

determination. It is useful to realise this because the determination of vitamin A in cod liver oils always involves the giving of extra vitamin D also, sometimes enough to double the amount of vitamin D that the rat is already receiving.

B. **The influence of an excess of vitamin B_1 on the determination of vitamin D.**

The increase in weight of rats during a vitamin D determination by the " line test " is often not very great. The reason for this has never been fully investigated. It has generally been assumed that 76% of yellow maize, freshly ground, provided sufficient vitamin B (complex) for the young growing rat, and that the depressing effect of the diet was due to its high calcium, low phosphorus ratio. However, Bruce and Phillips investigated the effect on a vitamin D determination, of giving rats large doses of vitamin B_1.

The rats were prepared as for an ordinary vitamin D determination by giving them the rachitogenic diet (Steenbock's 2965) for 3 weeks. Then a half of each litter was given 0·15g. or 0·10g. per rat per day of a vitamin B_1 preparation (kindly supplied by Professor Jansen) to make a total of 100 units (1·0g.) during the 10 days' test period. The other half of each litter was given no extra vitamin B_1. Each rat of every litter was given 10 International units of vitamin D at the beginning of the 10 days' test period. At the end of that time the rats were killed and healing assessed in the usual way. There was no significant difference in the average healing of the two groups of rats (Table XIII). Thus it was shown that excess of vitamin B_1 has no influence on the determination of vitamin D by the " line test."

TABLE XIII

TO SHOW THAT EXCESS OF VITAMIN B_1 DOES NOT INFLUENCE THE RESPONSE TO A DOSE OF VITAMIN D

	No. of rats.	Average healing.	Average increase in weight in 10 days, g.
Rats given 100 units vitamin B_1 and 10 units vitamin D	12	2·68	5·7
Rats given 10 units of vitamin D only	12	2·50	4·4

C. **The influence of an excess of vitamin A on the determination of vitamin D.**

The amount of vitamin A present in yellow maize which constitutes 76% of Steenbock's rachitogenic diet (2965) is more than sufficient for very rapid growth of the rat. This was shown by Bruce and Phillips, who found that a diet consisting of

Yellow maize	70%
Wheat gluten	18%
Dried yeast	8%
Salt mixture (Steenbock's 40)	4%

produced a rapid increase in weight in rats which had become steady in weight on the vitamin A-free diet of the laboratory. Seven male rats gained an average of 45·7g. and 7 female rats gained an average of 33·1g. in 3 weeks on the maize diet. The diet had been constituted to be as much like the Steenbock's 2965 diet as possible with the exceptions that (*a*) dried yeast was added to make sure that a possible shortage of vitamin B (complex) should not limit the response, and (*b*) Steenbock's salt mixture was substituted for the calcium carbonate and sodium chloride of the rachitogenic diet, thus removing any possible depressing effect of the high-calcium, low-phosphorus content of the diet and giving the rats one of the best salt mixtures known. The result showed that 70% of yellow maize provided ample amounts of vitamin A, possibly even excessive amounts. More vitamin A than this would certainly be in excess of requirements.

An experiment was therefore carried out on two groups of 11 animals each, each litter of rats being divided equally between the two groups. The rats were prepared as for a vitamin D determination and at the beginning of the test period all of them were given 10 units of vitamin D. During the test period each rat of one group was given a supplement of 40 units of vitamin A as carotene, per day, for the first 9 days. The rats of the other group were given no other supplement. At the end of the test period the examination of the bones was carried out as in the " line " test. The average healing in the rats given the extra vitamin A as carotene was 1·64 and the average healing of the rats given no extra vitamin A was 2·09. This appeared to indicate that the excess carotene had a depressing influence on the effect of vitamin D. A statistical

examination of the results indicated that there was only about a 4·5% chance (*i.e.* 1 in 22) of the difference being "due to random sampling" and therefore the difference was probably significant. The test was therefore repeated on 15 pairs of rats, the same supplements being given as before. This time the carotene appeared to enhance the effect of the vitamin D slightly, the average healing in the 15 rats given the extra carotene being 2·02 and that of the rats given no carotene being 1·80. The figures showed that there was a 29% chance of this difference being due to random sampling. Since one test appeared to show that carotene given in addition to a diet already containing abundance of it depressed the action of vitamin D and a second test appeared to show that a similar addition to the same diet increased the action of vitamin D, and also since there was a very fair chance of both differences being insignificant it must be concluded that excess of vitamin A (as carotene) has no influence on the action of vitamin D.

It therefore appears (*a*) that a partial deficiency of one vitamin in a diet which is used during the determination of another vitamin will influence the result however the test may be arranged; and (*b*) that an excess of any one vitamin in a diet used during the determination of another vitamin will not influence the result. Thus one need never be afraid of giving too much of the vitamins of the basal diet; the danger lies in giving too little.

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PART II

CHAPTER VIII

THE STANDARD DEVIATION

As determinations of vitamins become more and more carefully carried out, the more evident does it become that the response of different animals to apparently similar treatment varies greatly, and that this variation in response is uncontrollable. A simple example of this variation is seen in the increase in weight of the young rats of a colony that have been fed on a diet as nearly uniform as it could be made for many years. Not only does the average increase in weight in a given time vary between different litters but the increase in weight of members of the same litter varies. It is not surprising therefore to find the responses of different animals to equal doses of a vitamin varying in a similar way. Doubts arise as to how much faith can be placed in the averages of results which vary as vitamin results vary. The average of one group of results may be twice as great as the average of another group, but the variation in the results within each group may be so great that it is at least questionable, whether the averages are significantly different or not. The following chapters have been written in the hope that they may help students to determine, by recognised statistical methods, to what extent they can believe in their results, assuming, of course, that their technique is as sound as careful scientific training can make it.

1. The Standard Deviation of a Single Observation, σ

When a number of animals have received the same treatment the result of that treatment is usually stated as the average of the results obtained with the different animals, but

the average gives no indication of the range of variation of the different results. A certain calculation can, however, be made from these results, which expresses in a recognised form the range of the variation. The figure obtained by this calculation is called the standard deviation of a single observation. It is usually denoted by the Greek letter σ. It gives the limits on each side of the mean, within which two-thirds of the results (or observations) lie, and outside of which one-third of the results lie. This proportion must not be expected exactly when the number of animals (or observations) is small. The larger the number of animals used the more nearly will the spread of the results approach this proportion. If the standard deviation has been determined from a sufficiently large number of animals it is allowable to predict that if the same experiment is performed on a further large number of animals, then the same proportion of the results from the second batch of animals will lie between the same limits. From this figure for the standard deviation, the proportion of results lying within (or outside) any other limits can be calculated. For example, half the results or observations will lie within the mean plus or minus two-thirds of σ, *i.e.* within $M \pm \frac{2}{3}\sigma$, and the other half will lie outside these limits. Similarly, 95·5% of the observations will lie within $M \pm 2\sigma$ and 4·5% (one-twenty-second) outside these limits, and 99% of the results will lie within $M \pm 2{\cdot}576\sigma$.

It follows then, that when the standard deviation of a certain type of observation has been determined from a large number of these observations, that there is one chance in three that the next single observation will lie outside the mean plus or minus the standard deviation. If there is one chance in three that the next single observation will lie outside $M \pm \sigma$, then there is one chance in six that it will be greater than $M + \sigma$ and one chance in six that it will be less than $M - \sigma$.

Similarly, there is a one-in-two chance that any single observation will lie outside $M \pm \frac{2}{3}\sigma$, and a one-in-twenty-two chance (approximately 4·5%) that it will lie outside $M \pm 2\sigma$. Tables have been constructed denoting the probability of a result lying outside any other limits (Fisher, *Statistical Methods for Research Workers*, 6th Edition, 1936, p. 80).

Example.—One hundred female rats of a stock colony made the increases in weight shown in column 2 of Table XIV during

the period between the day they were 5 weeks old and the day they were 10 weeks old. They were the first 100 rats put to breeding, June 1930 to March 1931, from the second generation of a colony fed on a particular diet in the writer's laboratory. They were not selected in any way from the mass of rats put to breeding. The average (or mean) increase in weight during this 5 weeks' period was 56·6g. To find the standard deviation, σ, of a single observation from these 100 results,

(1) write down all the observations in a single column (column 2 of Table XIV) and calculate the average of the 100 observations (=M or $\bar{x}$) ;

(2) subtract the average value from each observation $(x-\bar{x})$ and put down the deviations from the mean in two columns, positive and negative; check the arithmetic by summing the positive deviations and the negative deviations separately and see that these totals agree (column 3) ;

(3) square each deviation and put down the squares, $(x-\bar{x})^2$, in the next column (column 4). These values are all positive ;

(4) add up the squares of the deviations $=\Sigma(x-\bar{x})^2$;

(5) divide the sum of the squares by the figure which is less by one than the number of observations $(n-1)$. When the number of animals is small $n-1$ is used as the divisor ; when the number is large n is used although then it is not very different from $n-1$. For all animal experiments use $n-1$. This result gives the "variance" $=\dfrac{\Sigma(x-\bar{x})^2}{n-1}$;

(6) take the square root of the variance. This result is the standard deviation, σ, of a single observation. Some workers use the letter, s, to denote the standard deviation calculated from a limited number of observations, and reserve σ for the true value which would have had to be calculated from an unlimited number of observations.

TABLE XIV

CALCULATION OF THE STANDARD DEVIATION OF A SINGLE OBSERVATION ON THE INCREASE IN WEIGHT OF FEMALE RATS FROM THE END OF THE 5TH WEEK TO THE END OF THE 10TH WEEK OF LIFE.

Rat.	Increase in weight from 5th to 10th week of life, x.	Difference between increase in weight of each rat and the average increase in weight ($\bar{x}$) $x-\bar{x}$		Square of difference (or deviation) from mean, $(x-\bar{x})^2$.
		+difference.	−difference.	
1	55		1·6	2·56
2	58	1·4		1·96
3	56		0·6	0·36
4	56		0·6	0·36
5	61	4·4		19·36
6	60	3·4		11·56
7	25		31·6	998·56
8	48		8·6	73·96
9	56		0·6	0·36
10	54		2·6	6·76
11	51		5·6	31·36
12	59	2·4		5·76
13	43		13·6	184·96
14	60	3·4		11·56
15	49		7·6	57·76
16	48		8·6	73·96
17	48		8·6	73·96
18	54		2·6	6·76
19	52		4·6	21·16
20	21		35·6	1,267·36
21	33		23·6	556·96
22	33		23·6	556·96
23	63	6·4		40·96
24	70	13·4		179·56
25	64	7·4		54·76
26	70	13·4		179·56
27	61	4·4		19·36
28	47		9·6	92·16
29	50		6·6	43·56
30	61	4·4		19·36
31	59	2·4		5·76
32	59	2·4		5·76
33	48		8·6	73·96
34	74	17·4		302·76
35	58	1·4		1·96
36	46		10·6	112·36
37	51		5·6	31·36
38	48		8·6	73·96
39	58	1·4		1·96
40	35		21·6	466·56
41	69	12·4		153·76
42	72	15·4		237·16
43	47		9·6	92·16
44	42		14·6	213·16
45	39		17·6	309·76
46	35		21·6	466·56
47	36		20·6	424·36
48	62	5·4		29·16
49	76	19·4		376·36
50	72	15·4		237·16

Rat.	Increase in weight from 5th to 10th week of life, x.	Difference between increase in weight of each rat and the average increase in weight ($\bar{x}$). $x-\bar{x}$.		Square of difference (or deviation) from mean, $(x-\bar{x})^2$
		+difference.	−difference.	
51	74	17·4		302·76
52	62	5·4		29·16
53	51		5·6	31·36
54	65	8·4		70·56
55	67	10·4		108·16
56	58	1·4		1·96
57	25		31·6	998·56
58	62	5·4		29·16
59	47		9·6	92·16
60	41		15·6	243·36
61	29		27·6	761·76
62	55		1·6	2·56
63	33		23·6	556·96
64	54		2·6	6·76
65	72	15·4		237·16
66	69	12·4		153·76
67	53		3·6	12·96
68	65	8·4		70·56
69	64	7·4		54·76
70	63	6·4		40·96
71	68	11·4		129·96
72	77	20·4		416·16
73	59	2·4		5·76
74	52		4·6	21·16
75	79	22·4		501·76
76	48		8·6	73·96
77	71	14·4		207·36
78	76	19·4		376·36
79	66	9·4		88·36
80	66	9·4		88·36
81	54		2·6	6·76
82	33		23·6	556·96
83	55		1·6	2·56
84	60	3·4		11·56
85	57	0·4		0·16
86	53		3·6	12·96
87	43		13·6	184·96
88	67	10·4		108·16
89	53		3·6	12·96
90	47		9·6	92·16
91	62	5·4		29·16
92	64	7·4		54·76
93	74	17·4		302·76
94	71	14·4		207·36
95	68	11·4		129·96
96	74	17·4		302·76
97	77	20·4		416·16
98	73	16·4		268·96
99	69	12·4		153·76
100	83	26·4		696·96
Total	5,660	+528·2	−528·2	17,476·00
Mean	56·6=$\bar{x}$	=0		=$\Sigma(x-\bar{x})^2$

Then $$v=\frac{\Sigma(x-\bar{x})^2}{n-1}=\frac{17{,}476{\cdot}00}{100-1}=\frac{17{,}476{\cdot}00}{99}=176{\cdot}52$$

and $$r=\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}=\sqrt{176{\cdot}52}=\pm 13{\cdot}3$$

To test the value 13·3 of σ for the above set of observations: According to theory

(*a*) one-half of the observations should lie outside the values $M+\frac{2}{3}\sigma=65{\cdot}5$ and $M-\frac{2}{3}\sigma=47{\cdot}7$;

(*b*) one-third of the observations should lie outside the values $M+\sigma=69{\cdot}9$ and $M-\sigma=43{\cdot}3$;

(*c*) one-twenty-second of the observations should lie outside the values $M+2\sigma=83{\cdot}2$ and $M-2\sigma=30{\cdot}0$.

Count the number of observations that actually lie outside these limits and compare the numbers found with the numbers given by the theory.

	Number of observations outside the limits.	
	(*a*) According to theory.	(*b*) Actually found.
$M\pm\frac{2}{3}\sigma$	50·0	48
$M\pm\sigma$	33·3	32
$M\pm 2\sigma$	4·5	4

Considering the smallness of the number (n) of observations used in this example, the agreement between found and calculated results may be said to be very good. Such good agreement must not be expected in every group of a hundred animals examined.

The basis of the calculation

H. Poincaré wrote in the Preface to his *Thermodynamique* (Paris, 1892), "Un physicien éminent me disait un jour à propos de la loi des erreurs: 'Tout le monde y croit fermement parceque les mathématiciens s'imaginent que c'est un fait d'observation, et les observateurs que c'est un théorème de mathématiques.'"

Certainly when tested on large enough groups of animals which have been treated as much alike as possible, the observa-

tions have demonstrated the validity of the theory over and over again.

The distribution or scatter of observations is often depicted graphically. If the number of observations is large enough, a smooth curve is obtained. Suppose that, instead of the hundred rats in the example given above, there had been a thousand or ten thousand or, preferably, a million. If the number of rats which showed each increase in weight were plotted against those increases in weight, a curve such as that depicted in Fig. 38 would be obtained, provided the observations were indeed normally distributed, *i.e.* provided that no disturbing factor had been introduced which had made it not merely one set of observations but more.

In Fig. 38 the mean, M, of say, a million (or many millions)

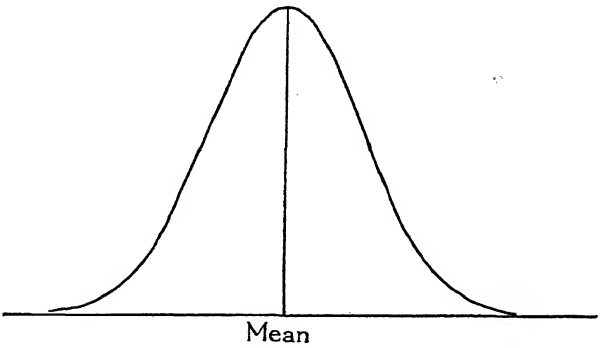


FIG. 38.—Normal curve of distribution.

observations has been placed as an abscissa at a convenient distance from 0. The number of observations exactly equal to M (*i.e.* the number of rats whose increases in weight were each equal to M) has been plotted against M. The number of observations exactly equal to M+1 and M—1 have been plotted against those respective points, the number equal to M+2 and M—2 against those, and so on until the point is reached theoretically beyond which no observations on the whole million were obtained. Then if all the observations of the values M, M$\pm$1, M$\pm$2, M$\pm$3, . . . M$\pm\frac{2}{3}\sigma$ (whatever σ has been found by calculation to be) are added together, they would amount to half a million, and if all the observations outside the limits M$\pm\frac{2}{3}\sigma$ were added together they would amount to half a million. Similarly, all the observations lying

outside $M \pm \sigma$ would add up to one-third of a million. All the observations lying outside $M \pm 2\sigma$ would add up to about 4·5% of the million. All the observations lying outside $M \pm 2{\cdot}576\sigma$ would add up to 1·0% of the million. The limits outside of which any other proportion of observations lie may be found from the appropriate table ("Table of x" in Fisher's *Statistical Methods for Research Workers*, 6th Edition, p. 80, 1936).

2. Test of Normalcy of the Distribution of Results

Sooner or later, workers on the determination of the vitamins must discover whether the variations in the results of their tests are indeed normally distributed; that is, whether they form a "normal" curve when enough results are available and they are plotted as in Fig. 38. This can be done by calculations much more satisfactorily than by plotting, for it is seldom that enough results are collected to form a really smooth curve. Two calculations are made:

(*a*) $\mu_3 = \frac{\Sigma(x-\bar{x})^3}{n}$, called the "third moment."

(*b*) $\mu_4 = \frac{\Sigma(x-\bar{x})^4}{n}$, called the "fourth moment."

The values of μ_3 and μ_4 are calculated in a way similar to the calculation of $\mu_2 = \frac{\Sigma(x-\bar{x})^2}{n}$ which is called the "second moment."

Then, the more nearly the value of β_1 which $= \frac{\mu_3^2}{\mu_2^3}$ approximates to the value 0, the more nearly is the curve symmetrical. The more nearly the value of β_2 which $= \frac{\mu_4}{\mu_2^2}$ approximates to the value of 3·0, the more nearly does the curve have the right height in proportion to its width.

Example.—In the writer's laboratory the variation in response to doses of vitamin A has been worked out by this method and found to be normal.

The number (n) of male rats in the group of tests used for this calculation was 922. Σd^2, Σd^3 and Σd^4 were calculated in the usual way. (See Chapter IX.)

Then, $$\mu_2=\frac{\Sigma d^2}{n}=\frac{+117{,}063}{922}=126{\cdot}97$$

$$\mu_3=\frac{\Sigma d^3}{n}=\frac{-175{,}283}{922}=-190{\cdot}11$$

$$\mu_4=\frac{\Sigma d^4}{n}=\frac{+41{,}492{,}207}{922}=45{,}002{\cdot}39$$

and $$\beta_1=\frac{{\mu_3}^2}{{\mu_2}^3}=\frac{(-190{\cdot}11)^2}{126{\cdot}97^3}=0{\cdot}018$$

therefore the curve is symmetrical.

$$\beta_2=\frac{\mu_4}{{\mu_2}^2}=\frac{45{,}002{\cdot}39}{126{\cdot}97^2}=2{\cdot}79$$

therefore the curve is slightly flat.

The number of female rats in the group used for this calculation was 1,174.

Then $$\mu_2=\frac{+92{,}965}{1{,}174}=79{\cdot}19$$

$$\mu_3=\frac{-277}{1{,}174}=-0{\cdot}24$$

$$\mu_4=\frac{+26{,}213{,}467}{1{,}174}=22{,}319{\cdot}82$$

and $$\beta_1=\frac{{\mu_3}^2}{{\mu_2}^3}=\frac{(-0{\cdot}24)^2}{79{\cdot}19^3}=0{\cdot}000000115$$

therefore the curve is symmetrical.

$$\beta_2=\frac{\mu_4}{{\mu_2}^2}=\frac{22{,}319{\cdot}82}{79{\cdot}19^2}=3{\cdot}5$$

therefore the curve is a little peaked.

Therefore the variation in response to doses of vitamin A may be said to be normal, or the deviations from the mean response may be said to be normally distributed.

3. The Standard Error of the Mean (ϵ) (sometimes called the Standard Deviation of the Mean)

Just as a figure can be obtained to express the probability that a single observation will lie outside certain limits on each side of the mean, so a figure can be obtained to express the probability that an average result from any given number of animals will lie outside certain limits on each side of the mean

result obtained from all the animals investigated. That is, if several hundred animals have been experimented upon, giving a value for σ which may be regarded as approaching the true value for σ, it is possible to calculate the chance that the average result of a small group of, say, 10 animals will lie outside certain limits on each side of the true mean M. The calculation is made from the standard deviation of a single observation by dividing it by the square root of the number of animals used, *i.e.* $\epsilon=\frac{\sigma}{\sqrt{n}}$.

Then, just as

50% of the single observations lie outside $M\pm\frac{2}{3}\sigma$
33·3% ,, ,, ,, $M\pm\sigma$
4·5% ,, ,, ,, $M\pm2\sigma$

So, if all the single observations are divided without selection into groups of a certain number of animals,

50% of the averages of the groups lie outside $M\pm\frac{2}{3}\epsilon$
33·3% ,, ,, ,, $M\pm\epsilon$
4·5% ,, ,, ,, $M\pm2\epsilon$

Example.—The mean increase in weight of the 100 rats from which the calculation on p. 154 was made was 56·6g., which may or may not have been near the true mean for the colony. The standard deviation of a single observation of the increase in weight was found to be ±13·3g. This means that one-third of the 100 rats made increases in weight outside the limits $M\pm\sigma$, *i.e.* greater than 69·9g. or less than 43·3g.

Now the standard error, ϵ, of the average result of a group of 10 of these rats is found by dividing the standard deviation by the square root of the number of animals in the group—

i.e. $$\epsilon=\frac{\sigma}{\sqrt{n}}=\pm\frac{13\cdot3}{\sqrt{10}}=\pm\frac{13\cdot3}{3\cdot16}=\pm4\cdot2\text{g.}$$

This means that, of all the groups of 10 animals,

$\frac{1}{2}$ of the groups should have a mean lying outside $56\cdot6\pm\frac{2}{3}\times4\cdot2$g.

$\frac{1}{3}$ of the groups should have a mean lying outside $56\cdot6\pm4\cdot2$g.

$\frac{1}{22}$ of the groups should have a mean lying outside $56\cdot6\pm2\times4\cdot2$g.

$\frac{1}{100}$ of the groups should have a mean lying outside $56\cdot6\pm2\cdot576\times4\cdot2$g.

This last corresponds to P=0·99, the limits of error adopted for specified numbers of pairs of animals in the 1936 Addendum to the British Pharmacopœia, 1932.

It is suggested that the student should now divide the 100 figures given in column 2 of Table XIV into ten groups of 10 rats each, taking them in sequence exactly as they occur in the table; then find the average of each group and see what proportions of the ten groups lie outside the expected limits for the groups. He should not expect a very good agreement between found and calculated proportions as he has only ten of these groups with which to work.

The above results may be stated in another way. When once the standard deviation of a single observation and from it the standard error of the mean of a group of 10 animals have been found, the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm \frac{2}{3}\epsilon$ is 1 in 2. Similarly, the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm \epsilon$ is 1 in 3, and the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm 2\epsilon$ is 1 in 22. Also the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm 2{\cdot}576\epsilon$ is 1 in 100.

It should be realised at this stage that since $\epsilon = \frac{\sigma}{\sqrt{n}}$ the accuracy of an average result is inversely proportional to the square root of the number of animals used, *e.g.* to halve the inaccuracy of an average result, four times the number of animals must be used; 36 animals are required to reduce to one-half the inaccuracy of an average result in which 9 animals were used, and using 100 animals is only twice as good as using 25 animals. This should be considered when a decision has to be made as to how many animals should be used in any experiment.

The "probable error," a term frequently used in biological estimations, is the value of $\frac{2}{3} \cdot \epsilon$, *i.e.* $\frac{2}{3} \cdot \frac{\sigma}{\sqrt{n}}$. It may be used in a general sense for a particular type of determination whose standard deviation has been determined from experiments on a very large number of animals; and then the "probable error" must be accompanied by a statement of the number of animals used, *e.g.* "the probable error of a test when a group of

10 animals is used, is . . ." The term may also be used for expressing the error of an experiment which has been performed only once on a group of, say, 5 animals, when the probable error of the mean of the five results would be found first by determining σ for that group of 5 animals, then dividing it by $\sqrt{5}$ and then multiplying it by $\frac{2}{3}$, *i.e.* the probable error of the mean was $\frac{2}{3}.\frac{\sigma}{\sqrt{5}}\left(=\frac{2}{3}\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}\times\frac{1}{\sqrt{n}}=\frac{2}{3}\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}\right)$

4. The Difference between Two Means

Workers often find such a divergency in the individual results in two groups of animals used in a comparison that they seriously doubt whether the apparent difference between the averages of the two groups really means anything or not, *i.e.* whether the difference between the two means is significant or not. A calculation can be made to determine this.

The standard deviation of the results given by each group of animals is determined, and from this the standard error of each group. These are substituted in the formula $\frac{x_1-x_2}{\sqrt{\epsilon_1{}^2+\epsilon_2{}^2}}$ in which x_1 is the average result of one group and ϵ_1 is its standard error, x_2 is the average result from the other group and ϵ_2 is its standard error. If on working out the value obtained by these substitutions the result is found to be greater than 3, the difference between x_1 and x_2 may be accepted as significant, *i.e.* there is a good probability that a real difference exists. Some workers are indeed satisfied if they find the value to be greater than 2. This is a matter of temperament. It really means that if the result is greater than 3 there is a stronger chance of the difference being significant than if the result is greater than 2 and less than 3.

The statement written above may be expressed thus: If the difference between the means is more than three times the square root of the sum of the squares of the standard errors of the groups used, then this difference is significant.

5. References

Addendum 1936 to the British Pharmacopœia, 1932. The General Medical Council, London.

Fisher. Statistical Methods for Research Workers, 6th Edition, 1936. Oliver and Boyd, Edinburgh and London.

CHAPTER IX

THE ACCURACY OBTAINABLE IN DETERMINATIONS OF VITAMIN A

1. The Influence of the Slope of the Curve of Response on the Accuracy of a Determination

It has been explained in Part I that the potencies of two doses of vitamin A are not directly proportional to the average increases in weight which are brought about in the rats by those doses. The relationship between dose and effect is a curvilinear one. Therefore the probable error of the result of giving a particular dose of vitamin A to 10 rats cannot be obtained by determining the ratio between $M-\frac{2}{3}\epsilon$, M, and $M+\frac{2}{3}\epsilon$, or by stating $M-\frac{2}{3}\epsilon$ and $M+\frac{2}{3}\epsilon$ as a percentage of M. It is necessary to find the abscissæ corresponding to M, $M+\frac{2}{3}\epsilon$ and $M-\frac{2}{3}\epsilon$ from the curve relating response to dose given and then find the ratio of these abscissæ, or express the abscissæ corresponding to $M\pm\frac{2}{3}\epsilon$ as percentages of the abscissa corresponding to M. The abscissæ may be determined directly from the curve drawn on graph paper or by calculation from the equation representing the curve.

Example.—Suppose the average increase in weight of 10 male rats given a dose of vitamin A daily for 5 weeks was 20g., and the standard deviation of the increase in weight was ±15g. Then the standard error using 10 rats would be $\epsilon = \pm\frac{15}{\sqrt{10}} = \pm 4{\cdot}75$g., and the probable error would be $\frac{2}{3}\epsilon$ which is ±3·17g. Suppose the curve of response for tests of this kind was $y = 10 + 50 \log. x$. Then the abscissæ of this curve corresponding to $y = M = 20$g., $y = M + \frac{2}{3}\epsilon = 23{\cdot}17$g. and $y = M - \frac{2}{3}\epsilon$

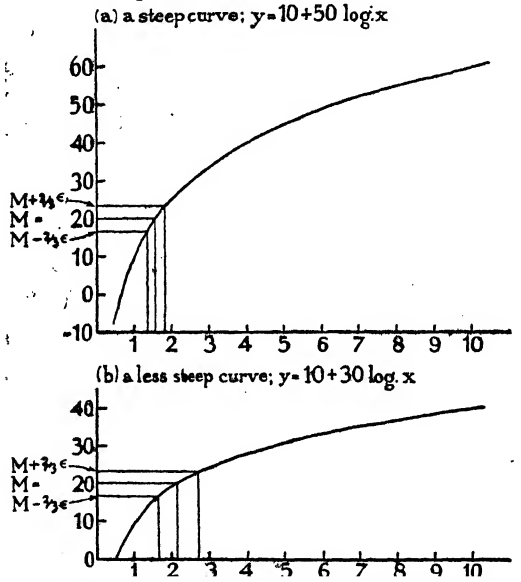


FIG. 39.—To find the probable error of a result from $\frac{2}{3}\epsilon$ and the curve of response.

=16·83g. are respectively 1·58, 1·83 and 1·36, from which the percentage error is calculated to be +16% or −13% (Fig. 39 (*a*)).

Suppose, however, that the curve of response was represented by the equation $y = 10 + 30 \log. x$. The calculation made from the same results, but with the use of this less steep curve, shows the abscissæ corresponding to M, $M + \frac{2}{3}\epsilon$, $M - \frac{2}{3}\epsilon$ to be 2·14, 2·75 and 1·69 respectively, and that the probable error of the result is +28% or −21% (Fig. 39 (*b*)).

It is evident that the steeper the curve of response relating

effect to dose given, the more accurate (or less inaccurate) is the result. Thus the accuracy of a biological test depends on two factors: (*a*) the standard error (which combines the standard deviation and the number of animals used), and (*b*) the steepness of the curve of response. The greater the standard deviation the greater the error, the greater the number of animals used the smaller the error, and the steeper the curve of response the smaller is the error.

2. The Influence of the Sex of the Experimental Animals on the Accuracy of a Determination of Vitamin A

The standard deviation of the increase in weight of male rats in vitamin A tests is greater than that of female rats. The curve of response of male rats to doses of vitamin A is steeper than that of female rats.

Few workers can have tested one particular dose of vitamin A on enough rats at one time to obtain a good idea of the value of the standard deviation of the test. The average of the standard deviations of many different tests suggests itself as a fair approximation to the true value of σ, but a better method is available. Each test is calculated separately as far as the summing of the squares of the deviations from the mean. That all the tests have different means does not matter when curves of response are logarithmic. (The student should satisfy himself on this point, either by theoretical considerations, or by making two or three other calculations as in Section I of this chapter with different values for M.) Then all these sums of the squares of the deviations are themselves added together and divided by the number of rats used minus the number of tests from which the calculation was made. This is, of course, equivalent to dividing by the sum of all the $(n-1)$'s of the different tests. The result of this gives the total variance of the estimation and the square root of the variance gives the standard deviation of the increase in weight of a single rat in this estimation.

In this way Coward (1932) determined the standard deviation of the response of male and female rats to doses of vitamin A given daily for 5 weeks after they had become steady in weight on a diet deficient in vitamin A (Table XV).

TABLE XV

	No. of animals.	No. of tests.	Standard deviation of a single observation.
Male rats ..	635	108	14·72
Female rats	672	108	11·02

The curves of response of young male and female rats to doses of vitamin A obtained by Coward, Key, Dyer and Morgan (1931) have been described in Chapter III. For a 5 weeks' test they were found to be:

for males $y=11{\cdot}3+50{\cdot}3 \log. x$

for females $y=12{\cdot}4+27{\cdot}4 \log. x$

where y=the increase in weight in 5 weeks

x=the dose of a particular sample of cod liver oil given daily.

Then, to determine the accuracy obtainable with male and female rats respectively in this test calculations with, say, a mean increase in weight of 20g. may be made as in the method described at the beginning of this section. The result is summarised in Table XVI. With the use of 10 male rats the probable error of the result is +15·4 or −13·4%. With the use of 10 female rats the probable error of the result is +21·7 or −17·5%.

TABLE XVI

THE PROBABLE ERROR OF THE MEAN RESULT OBTAINED BY GIVING A DOSE OF VITAMIN A TO A SINGLE GROUP OF (*a*) 10 MALE RATS AND (*b*) 10 FEMALE RATS.

	Increase in weight, g.	Abscissa corresponding to the increase in weight.	Abscissa expressed as a percentage of the mean.	Probable error of the result.
Male rats ..	M=20·0	1·49	100·0	—
	M+$\frac{2}{3}\epsilon$=23·11	1·72	115·4	+15·4%
	M−$\frac{2}{3}\epsilon$=16·89	1·29	86·6	−13·4%
Female rats	M=20·0	1·89	100·0	—
	M+$\frac{2}{3}\epsilon$=22·32	2·30	121·7	+21·7%
	M−$\frac{2}{3}\epsilon$=17·68	1·56	82·5	−17·5%

Thus, although male rats give results with a greater standard deviation than that given by female rats, the steeper slope of their curve of response causes them to give results of greater

accuracy than those given by female rats. (It is suggested here that as a useful exercise the student should (*a*) draw graphs to show how far the accuracy of a result in this test can be increased by increasing the number of animals used, and (*b*) determine how many female rats are required to give a result of the same accuracy as, say, 10 male rats.)

The curves of response relating the increase in weight in 5 weeks of male and female rats were first calculated from figures which included animals that lived more than $4\frac{1}{2}$ weeks, *i.e.* the dead weight of an animal which died within 3 days of the end of the test was included. In a later calculation, based on those rats only which were alive at the end of the fifth week of the test, the curves of response were found to be $y=13{\cdot}36+45{\cdot}18 \log. x$ (male rats) and $y=12{\cdot}55+27{\cdot}00 \log. x$ (female rats).

3. The Logarithmic Variance of a Result

The standard deviation divided by the slope of the curve of response gives a measure of the accuracy of the test called the standard deviation of the logarithm (to the base 10) of the result. It is designated by the Greek letter λ and the logarithmic variance by λ^2. Obviously, the greater the standard deviation the greater the logarithmic variance; and the greater the slope of the curve of response the smaller is the logarithmic variance. The logarithmic variance thus combines both factors which influence the accuracy of the result.

The accuracy of vitamin A tests can easily be expressed by the logarithmic variance, λ^2, or by λ. The slope of the curve of response is given by the coefficient of $\log. x$ in the equation representing the curve of response. This figure is the tangent of the angle which the curve makes with the X axis when the increases in weight are plotted against the logs. of the doses instead of against the doses themselves. Thus the slope of the curve $y=11{\cdot}3+50{\cdot}3x$ is 50·3 and that of the curve $y=12{\cdot}4+27{\cdot}4x$ is 27·4. The values of λ for male and female rats respectively from figures already quoted are found thus:

	σ	Slope of curve of response.	$\lambda=\frac{\sigma}{\text{slope}}$.
Male rats	14·72	50·3	0·29
Female rats	11·02	27·4	0·40

The ratio of the values of λ obtained from male and female rats is thus 0·29 : 0·40, which is about 1 : $\sqrt{2}$, and since the standard error of a test is inversely proportional to the square root of the number of animals used, it is evident that twice as many female rats as male rats must be used in a test to get the same degree of accuracy.

4. The Accuracy obtainable in a Comparison between Two Substances, e.g. the International Standard of Reference and a Substance whose Vitamin A Content is to be Determined

The foregoing calculations of accuracy are applicable to the results obtained from single groups of animals. As a vitamin determination must always involve a comparison between two substances, one of which is generally a standard of reference, an estimate of the accuracy of the comparison must be made which takes into account the inaccuracy of the average response from the dose of Standard and also that from the dose of test substance. This is done by consideration of the formula

$$\sigma^2=\sigma_1{}^2+\sigma_2{}^2-2r\sigma_1\sigma_2$$

where σ^2 is the variance of the comparison,

$\sigma_1{}^2$ is the variance of one group of rats,

$\sigma_2{}^2$ is the variance of the other group of rats,

and r is the correlation between σ_1 and σ_2.

Since $r=0$ (because the variability of one group is not dependent upon the variability of the other) and $\sigma_1=\sigma_2$ if the general value of the test is applied to both groups, then

$$\sigma^2=2\sigma_1{}^2$$

and

$$\sigma=\sqrt{2}\sigma_1.$$

Thus the standard error of the difference between the mean results from two groups of rats is $\sqrt{2}$ times the standard error of the mean from one group. In working out the probable error, say, of a determination of the vitamin A potency of a substance in terms of the Standard of reference, the probable error of the mean result from a given number of animals should be multiplied by $\sqrt{2}$ and the calculation carried out as before, or as shown in the following example :

Example.—The vitamin A content of a sample of butter

was estimated by testing in the usual way for 3 weeks only, simultaneously with a test on a dose of the International Standard.

	Dose.	No. of rats.	Average increase in weight in 3 weeks, g.	Abscissa corresponding to the increase in weight.	Average of abscissæ.
Butter ..	0·1g.	5♂	19·7	2·84	3·23
		5♀	18·7	3·83	
International Standard for vitamin A	2 units	5♂	9·0	1·26	1·04
		5♀	6·8	0·83	

Thus $\frac{\text{the vitamin A potency of 0·1g. butter}}{\text{2 units of vitamin A}} = \frac{3·23}{1·04}$

Therefore 0·1g. butter contained 6·2 units of vitamin A.
Therefore the butter contained 62 International units of vitamin A per gram.

The accuracy of this result was determined as follows: Since σ^2 (the variance) for bucks in a 3 weeks' test

$$=11·86^2=143·09$$

and σ^2 (the variance) for does in a 3 weeks' test

$$=9·74^2=96·27$$

σ^2 for an equal number of bucks and does is

$$\tfrac{1}{2}(143·09+96·27)=119·69$$

Thus σ for an equal number of bucks and does is 10·94.

The slope of the curve of response for a 3 weeks' test for male rats is 29·62.

The slope of the curve of response for a 3 weeks' test for female rats is 17·93.

Then the slope of the curve of response for an equal number of male and female rats is $\tfrac{1}{2}(29·62+17·93)=23·77$.

I.e. the curve has the form $y=a+23·77 \log. x$.

Thus the standard deviation of the log. of the result is $\frac{10·94}{23·77}$, and the standard error for 10 rats (5 male and 5 female) is

$$\frac{10·94}{23·77}\times\frac{1}{\sqrt{10}}$$

and the standard error of a comparison in which 10 rats (5 male and 5 female) were used for the test substance and a similar group for the Standard is $\frac{10{\cdot}94}{23{\cdot}77} \times \frac{1}{\sqrt{10}} \times \frac{\sqrt{2}}{1} = \pm 0{\cdot}206$. Hence the probable error of the log. of the result is

$$\pm\tfrac{2}{3} \times 0{\cdot}206 = \pm 0{\cdot}137$$

The probable error, then, of a determination of the vitamin A content of a substance when a simultaneous test is made on the Standard and the substance with 10 pairs of rats is given by the antilog. of $\pm 0{\cdot}137$ which is $+1{\cdot}37$ or $0{\cdot}73$, and the result may be outside 137 or 73% of the true result 1 out of 2 times (probable error).

5. The Accuracy of a Determination which has been made without the Use of a Previously Constructed Curve of Response or any General Estimate of the Standard Deviation of the Test

Occasionally there is enough available information on the possible potency of a product to arrange the dosing so that the result from one dose of the test substance may lie somewhere between the results obtained from two doses of the Standard of reference. If this information is not available a preliminary test will probably give it. (See Chapter III, Part I.) When that has been done the results from the two doses of Standard may be plotted against the logarithms of the doses given and a straight line drawn between them, *i.e.* it may be assumed that the curve is logarithmic in shape. The standard deviation is calculated from the three groups of rats and the accuracy determined as described for vitamin D in Chapter XII, p. 201.

If many tests are carried out in this way, the slopes of the curves obtained from the different tests may be averaged. The average slope may give a truer estimate of the slope of the curve of response than any one slope alone, and it would be possible to use the average slope, if desired, in further calculations rather than the slope obtained from a small number of animals in any one test.

6. The Influence of the Duration of a Vitamin A Test on the Accuracy of a Determination

It was felt for many years that a vitamin A test should be carried out for at least 5 weeks in order to get a fairly accurate

result. Some workers preferred an 8 weeks' test, others a 10 weeks' test. The method of determining the accuracy of a test described in the last few pages was used by Coward (1933) to determine how far the accuracy of a test could be increased by increasing the duration of the test. The tests which had supplied figures for the first calculation of the standard deviation of results from a 5 weeks' test, together with nearly as many tests carried out after the calculation was made, supplied the material for the calculations of the standard deviations of results for the 1, 2, 3, 4 and 5 weeks' tests. The 5 weeks' curves were recalculated, using only those rats which

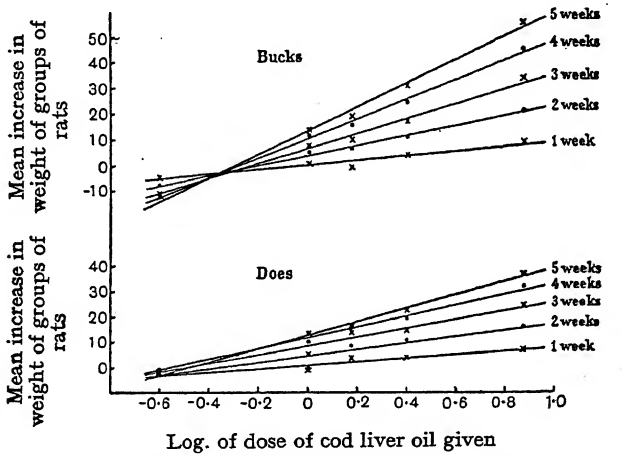


FIG. 40.—Curves of response relating increase in weight in 1, 2, 3, 4 and 5 weeks respectively to the logarithm of the dose of vitamin A given.

were alive at the end of the 5 weeks, which was considered preferable to including those which lived more than $4\frac{1}{2}$ weeks and whose dead weights had previously been included (Fig. 40).

Table XVII is extended beyond the table already published to include the probable error of a determination in which a comparison of one substance with another (generally the Standard) is made, using two groups of 10 rats each.

It is evident that the longer the test is carried on the greater is the accuracy obtained, but the relation between accuracy and duration of test is *curvilinear*, which was to be expected since it would require an infinite number of rats to reduce the standard error to zero. But the interesting point about these figures is that the inaccuracy falls considerably

from the 1 week's test to the 2 weeks' test, but not so much from the 2 weeks' test to the 3 weeks' test. The fall in inaccuracy from the 3 weeks' test to the 4 weeks' test is slightly less still, while the fall from the 4 weeks' test to the 5 weeks' test is only very slightly less. These figures have been depicted graphically in Fig. 41. It is, of course, a matter for each worker to decide for himself how long he considers it

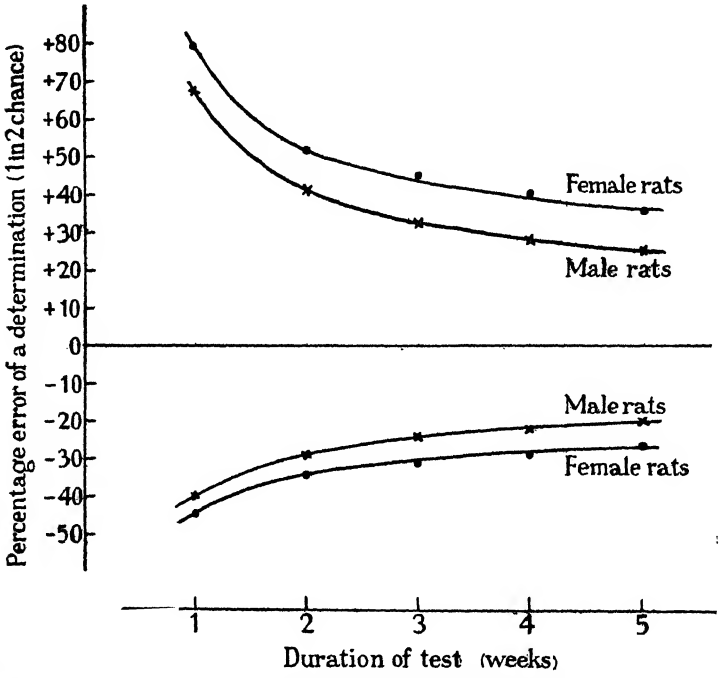


FIG. 41.—The probable error, expressed as a percentage, of a determination of vitamin A when 10 rats are used for the Standard and 10 for the substance tested, when the comparison is carried on for 1, 2, 3, 4 and 5 weeks respectively.

worth while to carry on a test, but Coward decided that for most tests a period of 3 weeks was long enough under the conditions in her laboratory.

Morgan (1934) claimed from a similar treatment of his results to have obtained a higher degree of accuracy in vitamin A tests than Coward had. He confirmed her finding that a 3 weeks' test was only slightly less accurate than a 5 weeks' test.

TABLE XVII

THE INCREASE IN ACCURACY OBTAINED BY PROLONGING THE DURATION OF A TEST FROM 1 TO 5 WEEKS

	σ.	Curve of response.	Slope of curve.	$\lambda = \frac{\sigma}{\text{slope}}$.	$\frac{2}{3} \cdot \frac{\lambda}{\sqrt{10}} \cdot \sqrt{2}$.	Range of probable error of determination.	Percentage range of probable error of determination.
Male rats—							
1 week's test	6·82	$y = -0{\cdot}33 + 9{\cdot}05 \log. x$	9·05	0·75	±0·2236	1·6734 0·5976	+67 −40
2 weeks' test	9·68	$y = 3{\cdot}50 + 19{\cdot}28 \log. x$	19·28	0·50	±0·1491	1·4096 0·7094	+41 −29
3 weeks' test	11·86	$y = 6{\cdot}04 + 29{\cdot}62 \log. x$	29·62	0·40	±0·1193	1·3161 0·7598	+32 −24
4 weeks' test	13·70	$y = 10{\cdot}20 + 38{\cdot}07 \log. x$	38·07	0·36	±0·1074	1·2806 0·7809	+28 −22
5 weeks' test	15·32	$y = 13{\cdot}36 + 45{\cdot}81 \log. x$	45·81	0·33	±0·0984	1·2542 0·7973	+25 −20
Female rats—							
1 week's test	5·63	$y = 0{\cdot}94 + 6{\cdot}64 \log. x$	6·64	0·85	±0·2537	1·7935 0·5576	+79 −44
2 weeks' test	8·05	$y = 4{\cdot}10 + 13{\cdot}45 \log. x$	13·45	0·60	±0·1789	1·5097 0·6624	+51 −34
3 weeks' test	9·74	$y = 8{\cdot}24 + 17{\cdot}93 \log. x$	17·93	0·54	±0·1610	1·4488 0·6902	+45 −31
4 weeks' test	11·11	$y = 11{\cdot}43 + 22{\cdot}48 \log. x$	22·48	0·49	±0·1461	1·3999 0·7143	+40 −29
5 weeks' test	11·93	$y = 12{\cdot}55 + 27{\cdot}00 \log. x$	27·00	0·44	±0·1312	1·3527 0·7392	+35 −26

7. The Accuracy Obtainable in Determinations in which Criteria other than "Increase in Weight" have been used

Any biological effect than can be measured more accurately than an "all or none" reaction lends itself to the same method for estimating its accuracy as the "increase in weight" method. The variation in response to a given dose must be calculated and a curve of response relating the effect to graded doses of the vitamin given must be constructed. If the curve of response proves to be logarithmic, then the calculation of the accuracy is made as in the "increase in weight" method. If it is not logarithmic different calculations of accuracy are made for results in the upper and lower parts of the curve. It should also be determined, if enough results are available, whether the standard deviation of the response to low doses is the same as that to high doses. If it is not, allowance must be made for this in the calculation. If there is not enough material to determine this the average standard deviation may be calculated in the usual way.

8. References

COWARD, 1932. Biochem. J., *26*, 691.
COWARD, 1933. Biochem. J., *27*, 445.
MORGAN, 1934. Biochem. J., *28*, 1178.

CHAPTER X

THE ACCURACY OBTAINABLE IN THE DETERMINATION OF VITAMIN B_1

1. The Determination of Vitamin B_1 by the Cure of Retracted Neck in Pigeons.
   - A. The percentage of birds cured.
     - (i) The standard error of the test.
     - (ii) The curve of response relating percentage of birds cured and dose of vitamin B_1 given.
     - (iii) The standard error of a comparison between two substances.
   - B. The duration of cure of retracted neck in pigeons.
2. The Determination of Vitamin B_1 by the Increase in Weight of Young Rats.
   - (i) The standard error of the test.
   - (ii) The accuracy obtainable in a comparison between two substances when a curve of response has previously been constructed.
   - (iii) The accuracy of a determination which has been made without the use of a previously constructed curve of response or any general estimate of the standard deviation of the test.
   - (iv) The standard deviation of the logarithm of the result.
   - (v) The accuracy of a vitamin B_1 determination in relation to the duration of the test.
3. References.

1. The Determination of Vitamin B_1 by the Cure of Retracted Neck in Pigeons

A. The percentage of birds cured.

(i) *The standard error of the test.*—The accuracy of a test which is based on an " all or none " reaction, *e.g.* the cure or the failure to cure retracted neck in a pigeon, is determined by a method which is somewhat different from the method employed for determining the accuracy of a test in which the amount or extent of the reaction can be measured.

In this test, as in all other biological tests, groups of animals are used for a determination, one group being given the Standard, and the others given the test substance. The percentage of animals in each group that react positively to

each dose is calculated. Then if n=the number of animals given the same dose of a substance, p=the percentage of animals that reacted positively, *e.g.* the number of pigeons in which retracted neck was cured, and q=the percentage of animals that reacted negatively, *i.e.* the number of pigeons in which retracted neck was not cured, the standard error of the average result of the group is

$$\epsilon = \sqrt{\frac{p.q}{n}}$$

Example.—Ten pigeons with retracted neck were given a dose of 0·03g. of the International Standard for vitamin B_1. Five of them were cured by the following morning, 5 of them were not cured. The standard error of the result was, therefore, given by the formula

$$\epsilon = \sqrt{\frac{p.q}{n}} = \sqrt{\frac{50 \times 50}{10}} = \sqrt{250} = \pm 15{\cdot}81$$

This means that if the test of 0·03g. of the International Standard were repeated on 10 pigeons with retracted neck a great many times, then, provided no influence caused the whole stock of pigeons to fluctuate in their response,

$\frac{1}{2}$ of the average results from the groups of 10 pigeons would lie outside $M \pm \frac{2}{3}\epsilon$, *i.e.* outside $50 \pm \frac{2}{3} \times 15{\cdot}81$, *i.e.* outside $50 \pm 10{\cdot}54$, *i.e.* would be either greater than 60·54% or less than 39·46%.

$\frac{1}{3}$ of the average results from the groups of 10 pigeons would lie outside $M \pm \epsilon$, *i.e.* outside $50 \pm 15{\cdot}81$, *i.e.* would be either greater than 65·81% or less than 34·19%.

$\frac{1}{22}$ of the average results from the groups of 10 pigeons would lie outside $M \pm 2\epsilon$, *i.e.* outside $50 \pm 31{\cdot}62$, *i.e.* would be either greater than 81·62% or less than 18·38%.

$\frac{1}{100}$ of the average results from the groups of 10 pigeons would lie outside $M \pm 2{\cdot}576\epsilon$, *i.e.* outside $50 \pm 40{\cdot}73$, *i.e.* would be either greater than 90·73% or less than 9·27%.

Similarly, when 60 or 40% of a group of 10 birds are cured the standard error of that result is

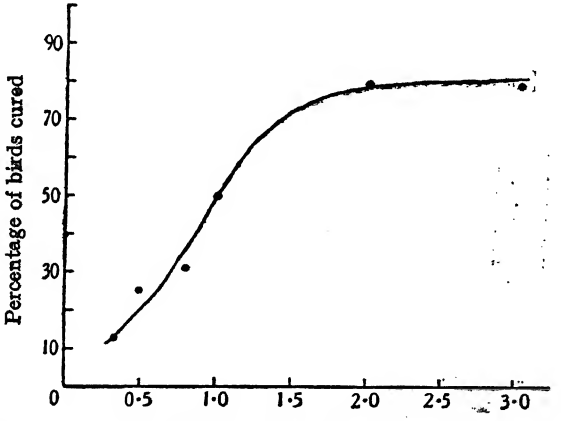
$$\epsilon = \sqrt{\frac{p.q}{n}} = \sqrt{\frac{60 \times 40}{10}} \text{ or } \sqrt{\frac{40 \times 60}{10}} = \pm 15{\cdot}49$$

and when 70 or 30% of a group of 10 birds are cured the standard error of that result is

$$\epsilon = \sqrt{\frac{p.q}{n}} = \sqrt{\frac{70 \times 30}{10}} \text{ or } \sqrt{\frac{30 \times 70}{10}} = \pm 14{\cdot}49$$

Thus the standard error of the result depends on the magnitude of the result itself; it is not independent of the result as it was in the increase in weight of determinations by rats in which the relationship of response to the dose given is logarithmic.

(ii) *The curve of response relating percentage of birds cured and dose of vitamin B_1 given.*—The curve of response of an "all or none" reaction is generally sigmoid, *i.e.* S-shaped. It rises slowly from the origin at first, then more steeply and then more and more slowly as 100% is approached. Often the



Arbitrary scale so chosen that 1·0 is the dose which cures 50% of birds

FIG. 42.—Curve of response relating percentage of birds cured and dose of vitamin B_1 given.

curve never reaches 100% at all. Coward, Burn, Ling and Morgan (1933) constructed a curve of response of pigeons to graded doses of vitamin B_1 from results gained with the International Standard of reference and with dried yeast (Fig. 42). This curve did not rise higher than 80% of birds cured, and at the upper end appeared to be nearly flat. By incorporating results from other tests, another curve of response was constructed (Fig. 43) which in the scale on which it was drawn is less obviously S-shaped and rises a little higher than the other curve but still does not reach 90% of birds cured, even with a dose four times as great as the dose that produced 70% of cures.

(iii) *The standard error of a comparison between two substances.* —When both doses (one of the Standard and one of the test substance) cure approximately 50% of the birds treated, the standard error of the result, 15·81 for 50% cures, is multiplied by $\sqrt{2}$ to obtain the standard error of the comparison. Then the abscissæ of the curve of response corresponding to

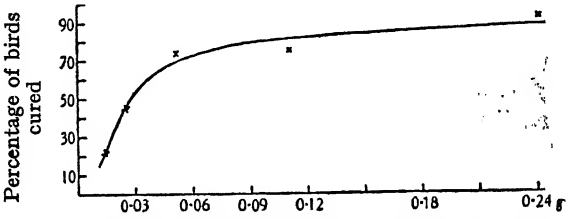


FIG. 43.—Curve of response relating percentage of birds cured to the dose of the International Standard (or equivalent) given.

$50 \pm \sqrt{2} \times 15{\cdot}81$, *i.e.* to 72·35 and 27·65 are found and the percentage error calculated. The "probable error" is calculated by finding the abscissæ corresponding to $\frac{2}{3} \times 15{\cdot}81 \times \sqrt{2}$, the 1 chance in 22 by finding the abscissæ corresponding to $2 \times 15{\cdot}81 \times \sqrt{2}$ and so forth (Table XVIII).

TABLE XVIII

THE ERRORS OF A DETERMINATION OF THE VITAMIN B_1 POTENCY WHEN THE DOSE TESTED AND THE DOSE OF STANDARD BOTH CURE APPROXIMATELY 50% OF THE BIRDS TREATED.

Probability.	Percentage range of errors.	Abscissa of Coward's curve of response corresponding to percentage of cures.	Percentage error of the determination.
1 chance in 2	50% cures	1·0	—
	$50+\frac{2}{3} \times 15{\cdot}81 \times \sqrt{2}=64{\cdot}90$	1·26	+26
	$50-\frac{2}{3} \times 15{\cdot}81 \times \sqrt{2}=35{\cdot}10$	0·79	−21
1 chance in 3	50% cures	1·0	—
	$50+15{\cdot}81 \times \sqrt{2}=72{\cdot}35$	1·48	+48
	$50-15{\cdot}81 \times \sqrt{2}=27{\cdot}65$	0·68	−31
1 chance in 22	50% cures	1·0	—
	$50+2 \times 15{\cdot}81 \times \sqrt{2}=94{\cdot}71$	too high for the curve	more than 100
	$50-2 \times 15{\cdot}81 \times \sqrt{2}=5{\cdot}29$	0·13	−87
1 chance in 100	50% cures	1·0	—
	$50+2{\cdot}576 \times 15{\cdot}81 \times \sqrt{2}=107{\cdot}57$	impossible	—
	$50-2{\cdot}576 \times 15{\cdot}81 \times \sqrt{2}=-7{\cdot}57$	impossible	—

It is suggested that the student should work out for himself at this stage the errors of a determination in which, say, 30% of the birds given a dose of Standard were cured and 30 or 70% of the birds given a dose of the other substance were cured.

The student should also consider how to estimate the error of a determination in which, say, 50% of the birds given a dose of Standard were cured and only, say, 30% of the birds given a dose of the other substance were cured.

Gaddum (1933) has solved this difficulty. Instead of plotting the percentage of birds cured as ordinates against the dose of vitamin B_1 given as abscissæ, he plots the " normal equivalent deviation " against the log. of the dose given. The " normal equivalent deviation," y, is a figure which expresses the error inherent in the percentage of positive reactions. When the percentage of, say, birds cured is 50, then $y=0$; when the percentage is 40 or 60, then $y=-0{\cdot}253$ or $+0{\cdot}253$ respectively; when the percentage is 30 or 70, then $y=-0{\cdot}524$ or $+0{\cdot}524$ respectively. Values for y corresponding to any other percentage of positive results may be found in Pearson's *Tables of Statisticians and Biometricians*, Part I, 3rd Edition, 1930, Table I, p. 1. The curve of response obtained in this way is, provided the distribution is normal, a straight line, or so nearly a straight line that it is permissible to construct the best straight line through the plotted points (as described in Part I, Chapter III). The slope of this curve, $y=a+bx$, is then represented by the coefficient of x, *i.e.* b. The standard deviation of the logarithms of the different effective doses, λ, is then estimated as the reciprocal of b, *i.e.* the distance on the logarithmic scale corresponding to a normal equivalent deviation of 1. Then, to allow for the positions on the curve on which the results fall, another factor, the " weight factor, B," has to be introduced. The factor corresponding to 50% birds cured is 0·637, to 40 or 60%, 0·625, to 30 or 70%, 0·578, to 20 or 80%, 0·49. It is found from the formula $\frac{1}{\sigma_y^2 n}$ in which σ_y is the sampling error of a percentile and is given by the formula $\sigma_y\sqrt{n}=\sqrt{pq}\times\frac{dy}{dp}$. The weight of an observation is equal to Bn.

Example.—Suppose a dose of

(*a*) 0·015g. International Standard produced 25% cures.
(*b*) 0·03g. International Standard produced 48% cures.
(*c*) 0·06g. International Standard produced 80% cures.

Then the curve of response is calculated as follows :

Percentage birds cured.	y.	Dose.	Log. of dose, x.	$x-\bar{x}$.	$y(x-\bar{x})$.	$(x-\bar{x})^2$.
25	−0·68	0·015	$\bar{2}$·1761	−0·3010	+0·20470	0·09061
48	−0·05	0·03	$\bar{2}$·4771	0	0	0
80	+0·84	0·06	$\bar{2}$·7782	+0·3010	+0·25287	0·09061
	3)0·11		3)$\bar{5}$·4314		Σ=0·45757	Σ=0·18122
	0·037		$\bar{x}=\bar{2}$·4771			

$$y=0\cdot037+2\cdot5(x-\bar{x})$$
$$=3\cdot8+2\cdot5x$$

$$b=\frac{0\cdot45757}{0\cdot18122}=2\cdot5$$

The slope of the curve is $b=2\cdot5$, and λ therefore $=0\cdot4$.

Suppose now that 10 pigeons have been dosed with 0·1g. of a sample of dried yeast and 10 other pigeons with a dose of 0·03g. of the International Standard and both groups have had 50% of cures. The potency of the dried yeast is calculated as $\frac{0\cdot03}{0\cdot1}$ of the potency of the International Standard, *i.e.* as $\frac{0\cdot03}{0\cdot1}\times100$ units=33 International units per gram.

The accuracy of this result is estimated as follows :

The standard deviation of the log. of the result is $\lambda=0\cdot4$.

The standard error of the log. of the result is $\frac{\lambda}{\sqrt{10}}=\frac{0\cdot4}{\sqrt{10}}$ and the standard error of the log. of the comparison between the 0·1g. dried yeast and 0·03g. Standard is $\frac{\lambda}{\sqrt{10}}\times\sqrt{2}=\pm0\cdot179$. This is the log. of 1·5101 or 0·6622. Hence there is 1 chance in 3 that the result will be more than 1·51 or less than 0·66 times the true result, *i.e.* that it may be more than 151% or less than 66% of the true result.

These figures confirm those in Table XVIII which were read directly from a curve drawn freehand through six points, three of which were the points which gave the curve of response

used in this calculation, and three were obtained from other experiments.

When the percentage of birds cured is not 50, then λ_M, the standard deviation of the logarithm of the ratio of the activities, may be determined from the formula

$$b^2\lambda_M{}^2=\frac{1}{S(B'n')}+\frac{1}{S(B''n'')}$$

where n' and n'' are the numbers of animals receiving the preparations being tested and B′ and B″ are the weight factors which depend on the percentage response of each group of animals.

B. The duration of cure of retracted neck in pigeons.

Coward, Burn, Ling and Morgan (1933) have published a curve of response relating the duration of the cure of retracted neck in the pigeon to the dose of vitamin B_1 given (Chapter IV, p. 58). They also estimated the standard deviation of the duration of cure from groups of pigeons of which all the members of any one group had received equal doses. The standard error of the estimation was calculated in the usual way.

The average standard deviation of a single determination of the duration of cure of retracted neck has more recently been estimated from 397 pigeons cured in 65 groups, by the method described for estimating the standard deviation of the increase in weight of rats in vitamin A determinations, *i.e.* by the formula

$$\sigma=\sqrt{\frac{\Sigma d^2}{N-M}}=\sqrt{\frac{3,710\cdot21}{397\cdot65}}=3\cdot34$$

where Σd^2 is the sum of all the squares of the deviations from the various means,

N=the number of pigeons,

M=the number of groups or means, *i.e.* N—M is the sum of all the $(n-1)$'s of the different groups.

Then the standard error of this test when 10 pigeons are used in a group is given by substituting in the formula

$$\epsilon=\frac{\sigma}{\sqrt{n}}=\frac{3\cdot34}{\sqrt{10}}=\pm1\cdot056$$

The " probable error " of the result from the group is

$$\tfrac{2}{3}\cdot\epsilon=\pm\tfrac{2}{3}\times1\cdot056=\pm0\cdot704$$

This, as usual, is converted into the probable error of the difference between the results obtained from two doses, by multiplying by $\sqrt{2}$. This gives 0·998 which can be converted into the percentage probable error of the determination by the calculation used in determinations of the error of other tests. Since the curve of response is nearly logarithmic, the percentage error can be calculated for any convenient mean, *e.g.* 4 days. The abscissæ corresponding to (*a*) the mean, M; (*b*) the mean plus the probable error, $M+\frac{2}{3}\epsilon$ (using 10 animals in each group); and (*c*) the mean minus the probable error, $M-\frac{2}{3}\epsilon$, are then determined from the curve and the percentage errors about the mean calculated (Table XIX).

TABLE XIX

THE PROBABLE ERROR OF A DETERMINATION OF VITAMIN B_1 AS JUDGED FROM THE DURATION OF CURE OF THE BIRDS, WHEN 10 PIGEONS HAVE BEEN CURED IN TESTING A DOSE OF THE STANDARD AND 10 PIGEONS HAVE BEEN CURED IN TESTING SIMULTANEOUSLY A DOSE OF THE PREPARATION UNDER TEST.

Average duration of cure.	Abscissa of Coward's curve corresponding to duration of cure.	Range of duration of cure, $\pm\frac{2}{3}\epsilon\times\sqrt{2}=\pm 0{\cdot}998$.	Range of abscissæ * corresponding to range of cure.	Percentage range of abscissæ.
4 days	0·049	4+0·998=4·998 4−0·998=3·002	0·074 0·032	151·0 65·3

2 The Determination of Vitamin B_1 by means of the Increase in Weight of Young Rats

(i) *The standard error of the test.*—The accuracy of this method for the determination of vitamin B_1 is calculated as it is in the "increase in weight" method of determinations of other vitamins. The standard deviation of a single observation must be determined, from which can be calculated the standard error for whatever size of group of animals is used, and a curve of response relating the increase in weight of the rats in a given time to the dose of vitamin B_1 given must be constructed (see Chapter IV, p. 59).

The standard deviation of a single observation is determined by the method described for vitamin A by the "increase in

* Obtained by direct reading from the curve.

weight " method, and also for vitamin B_1 by the " duration of cure " method used with pigeons. Each group of rats, of which all animals have been given equal doses of vitamin B_1, is treated in the usual way. The average increase (M) in weight of each group is found, the difference between each increase and the mean, *i.e.* the deviation (d) from the mean, and the squares of the deviations are calculated. The sum of the squares in each test is determined. Then all the sums of the squares of all the tests are added together and the standard deviation calculated by means of the formula

$$\sigma = \sqrt{\frac{\Sigma d^2}{N - M}}$$

where Σd^2=the sum of all the squares of the deviations from the various means,

N=the number of observations, *i.e.* of rats used,

M=the number of tests,

N—M is equal to the sum of all the $(n-1)$'s of the tests.

The standard deviation of male and female rats should be calculated separately.

The standard error for a group of, say, 10 rats, is obtained from the formula

$$\epsilon = \frac{\quad}{\sqrt{n}} = \frac{\quad}{\sqrt{10}}$$

(ii) *The accuracy obtainable in a comparison between two substances when a curve of response has previously been constructed.* —Coward, Burn, Ling and Morgan (1933) and Coward (1936) from further material, found that the standard deviations of male and female rats differed very little in this test and also that the curves of response of the male and female rats differed very little in slope. So they made a general estimate of the accuracy of their vitamin B_1 tests (rats) using the average standard deviation for male and female rats and the average curve of response, assuming equal numbers of male and female rats in each group. Furthermore, they found that the accuracy obtainable in vitamin B_1 tests on rats was much greater than that obtainable in either vitamin A or vitamin D tests. In the later tests, therefore, they used smaller groups of animals, *i.e.* 4 or 5 in a group rather than 10 to 12.

From 101 groups containing in all 282 male rats, the writer (1936) found a standard deviation of 8·16 on the increase in

weight in 3 weeks. For a group of 10 male rats the standard error is, therefore, 2·58. Since the potency of a substance is always obtained by testing a dose of the Standard at the same time as a dose of the substance, and both groups of rats show variations, the standard error of the accuracy of the result must be worked out on the standard error multiplied by the square root of 2 (see p. 166), *i.e.* $2{\cdot}58 \times \sqrt{2} = 3{\cdot}65$. The "probable error" is $\frac{2}{3}$ of $3{\cdot}65 = 2{\cdot}43$. The corresponding probable error for two groups of 10 female rats was found to be 2·21.

Since the curve relating the response of rats to doses of vitamin B_1 is logarithmic, the percentage error of the result can be calculated. A convenient value for M is chosen and, if the "probable error" of the estimation is wanted, the abscissæ corresponding to M, $M+\frac{2}{3}\epsilon \times \sqrt{2}$ and $M-\frac{2}{3}\epsilon \times \sqrt{2}$ are determined either directly from the curve or by substituting these values for y in the equation of the curve of response. The percentage probable error is then calculated from the abscissæ on either side of the abscissa corresponding to M, just as demonstrated for other vitamin determinations.

(iii) *The accuracy of a determination which has been made without the use of a previously constructed curve of response or any general estimate of the standard deviation of the test.*—The accuracy obtainable may, however, be calculated in another way, and this way can be used when no curve of response has been constructed previously and when only one or two doses of the preparation under test and two doses of the Standard have been tested.

The standard deviation of a single observation is calculated from the three or four groups available, male and female rats being calculated separately if desired, but the difference is so small that this is not necessary. The slope of the curve of response is determined from the responses to the doses of Standard, assuming the curve to be logarithmic and therefore plotting the responses against the logs. of the doses and joining the two points obtained. The slope b is obtained by substituting x_1y_1 of one point and x_2y_2 of the other point in the formula $\frac{y_2-y_1}{x_2-x_1}$. This gives the ratio between a difference in response and a difference in log. dose. An example of the calculation of the potency of a substance by this method was given in

Chapter IV, pp. 71–3. The calculation of the accuracy of the determination is made as described for vitamin D (Chapter XII, p. 201).

(iv) *The standard deviation of the log. of the result.*—This may be calculated in the usual way, *i.e.* by dividing the standard deviation, σ, by the slope of the curve relating increase in weight to the log. of the dose of vitamin B_1 given. To obtain the limits beyond which any proportion of results is likely to lie, multiply by the appropriate factor ($\frac{2}{3}$ for 50%, 1 for 33%, 2 for 4·5%, etc.); to obtain the error of the mean from a group of rats divide by the square root of the number of rats in the group; and to obtain the error of a determination in which two groups of rats are used (one for the test substance and one for the Standard) multiply by $\sqrt{2}$. Then take the antilog. of the answer and the error is obtained as a multiple or fraction of the true result. This method was used by the writer in the following calculation.

(v) *The accuracy of a vitamin* B_1 *determination in relation to the duration of the test.*—After some experience of determinations of the vitamin B_1 content of substances by the "increase in weight" method on rats, it seemed to the writer that a reasonable degree of accuracy might be obtained by cutting down the test or "curative" period from 3 to 2 weeks. The standard deviations of the increase in weight of male and female rats respectively in 1, 2 and 3 weeks were determined, and also the average slopes of the curves of response for 1, 2 and 3 weeks. The calculation of the probable errors of tests carried on for 1, 2 and 3 weeks were then determined as described above. The results are summarised in Table XX.

Thus, it is evident that almost as great accuracy is obtained by carrying on the test for only 2 weeks as if it had been carried on for 3 weeks. It is certainly preferable to carry on the test for 2 weeks rather than for only 1 week. The number of animals on which the calculation was based was 549 for the 1 week's test, 524 for the 2 weeks' test and 497 for the 3 weeks' test, some of the rats on the low doses having died during the course of the experiment.

Schultz and Knott reported (1936) an extensive investigation of the determination of vitamin B_1 by the "increase in weight" method with rats. They found that they obtained the least variation in response when (*a*) autoclaved whey was

TABLE XX

THE ACCURACY OBTAINABLE BY CARRYING ON THE TEST FOR 1, 2 OR 3 WEEKS WITH 10 PAIRS OF ANIMALS, ONE OF EACH PAIR HAVING BEEN GIVEN A DOSE OF THE STANDARD AND THE OTHER A DOSE OF TEST SUBSTANCE.

	σ.	Slope of curve.	$\lambda = \frac{\sigma}{\text{slope}}$.	$\frac{2}{3} \cdot \frac{\lambda}{\sqrt{10}} \cdot \sqrt{2}$.	Antilog. range of dose ratio.	Range of probable error of determination, %.
Male rats—						
1 week's test	4·31	20·70	0·208	0·0620 $\bar{1}$·9380	1·153 0·867	+15·3 −13·3
2 weeks' test	6·69	36·88	0·181	0·0539 $\bar{1}$·9461	1·132 0·883	+13·2 −11·7
3 weeks' test	8·16	52·76	0·155	0·0462 $\bar{1}$·9538	1·112 0·899	+11·2 −10·1
Female rats—						
1 week's test	4·99	17·54	0·284	0·0846 $\bar{1}$·9154	1·215 0·823	+21·5 −17·7
2 weeks' test	6·41	39·67	0·162	0·0483 $\bar{1}$·9517	1·118 0·895	+11·8 −10·5
3 weeks' test	7·43	56·01	0·133	0·0396 $\bar{1}$·9604	1·096 0·913	+ 9·6 − 8·7

substituted for autoclaved yeast and sucrose for dextrin in the basal diet, (*b*) rats of 60g. weight at the beginning of the preparatory period were used, and (*c*) the dose was so chosen that the average increase in weight of the rat over 10 days was from 1 to 2g. per day. Their curve of response relating effect to dose of vitamin B_1 given was a straight line when the log. of the average increase in weight per day was plotted against the log. of the dose given. They concluded that a 10 days' test period after the preliminary depletion period was long enough for a vitamin B_1 determination on rats.

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CHAPTER XI

THE ACCURACY OBTAINABLE IN THE DETERMINATION OF VITAMIN C

THE accuracy obtainable in a determination of vitamin C depends on the same two factors as that obtainable in a determination of vitamin A or vitamin D, *i.e.* (*a*) the standard deviation of a single observation and hence the standard error of the mean result from a group of animals, and (*b*) the steepness of the curve of response relating the magnitude of the effect to the dose of vitamin C given.

1. Key and Elphick's Modification of the Höjer Tooth Method

Key and Elphick (1931) published a curve of response to graded doses of vitamin C (Chapter V, p. 84), but up to the time of publication had not amassed sufficient data to make a reliable estimation of the standard deviation of their test. Since then, the method has been used without modification in the writer's laboratory where it was worked out and enough results have been collected to make a very fair estimate of the standard deviation of the test.

Nearly all the groups of animals given equal doses of vitamin C have proved to be of value for the determination of the standard deviation. The groups which were of no value were those in which no protection or almost no protection had occurred in the majority of the guinea-pigs and those in which most of the pigs had shown complete protection; for the scale for assessing protection did not provide figures less than 0 nor greater than 4. Hence the apparent variation in the groups at the ends of the curve would be less than the true variation.

Fifty-six groups, containing in all 274 guinea-pigs, were

available for the calculation of the standard deviation. Each group of results was treated as the groups of results in the vitamin A calculation. The mean of the group was determined, the difference (or deviation) of each result from the mean, the square of each deviation from the mean, and finally the sum of the squares of the deviations. All these sums of squares were themselves added and divided by the total number of guinea-pigs, N, minus the number of groups, M (*i.e.* by the sum of the $(n-1)$'s of the groups). This gave the variance of the test. The square root of the variance is the standard deviation of a single observation.

The value found for σ was 0·92. Then the standard error, ϵ, of a group of 10 guinea-pigs was $\frac{0\cdot92}{\sqrt{10}}=0\cdot29$ and the probable error of the mean of the results from 10 guinea-pigs was $\frac{2}{3}.\epsilon$ or $\frac{2}{3}.\frac{0\cdot92}{\sqrt{10}}$ which is 0·19. It is interesting to note that this value of σ, 0·92, was obtained from the first forty-one groups containing 173 animals, again when eleven groups containing 61 animals were added to the total, and again, finally, when four more groups containing 40 animals were added to the total. There was no evidence that the variation in response to high doses of vitamin C was different from that in response to low doses. To express the error as a percentage of the apparent value of the potency of the dose given a calculation is made similar to that used in the determination of vitamin A or vitamin D by means of the curve of response. But in the vitamin C test by the tooth method a difficulty presents itself. The curve of response in the tooth method was a straight line represented by the equation $y=0\cdot6+1\cdot15x$ (see Chapter V), and therefore, the percentage error, determined as it must be by the method described, must depend on whatever mean is chosen as the basis of the calculation. In biological tests generally, it is best to get a result midway between whatever stage is called 0 and the stage of complete healing or protection, for this allows for the greatest range of response on both sides of the mean. Therefore, for the calculation of the accuracy of an average result from a dose of vitamin C an amount of protection of 2·5 scale divisions has been chosen for the mean.

The probable error of a determination of the vitamin C content of a substance, made by comparing the effect of a dose

of the test substance on a group of n animals with that of a dose of the Standard on another group of n animals treated simultaneously, is found by multiplying the probable error of the mean of a group by $\sqrt{2}$ and then finding the abscissæ of the curve of response corresponding to M, $M \pm \frac{2}{3} . \epsilon . \sqrt{2}$, as in Table XXI.

TABLE XXI

THE PROBABLE ERROR OF A DETERMINATION OF THE VITAMIN C POTENCY OF A SUBSTANCE OBTAINED BY SIMULTANEOUS TESTS ON A DOSE OF THE SUBSTANCE AND A DOSE OF THE STANDARD, A GROUP OF 10 GUINEA-PIGS BEING USED FOR EACH. ($\sigma = 0{\cdot}92$, $y = 0{\cdot}6 + 1{\cdot}15x$. VALUES FROM THE PAPER BY KEY AND ELPHICK. PROBABLE ERROR OF COMPARISON $\frac{2}{3}\epsilon . \sqrt{2} = 0{\cdot}27$.)

Amount of protection afforded (scale divisions).	Abscissa corresponding to amount of protection.	Abscissa expressed as a percentage of the mean.	Probable error of the determination of vitamin C.
M $= 2{\cdot}5$	1·65	100	—
$M + \frac{2}{3} . \epsilon . \sqrt{2} = 2{\cdot}77$	1·89	114	+14%
$M - \frac{2}{3} . \epsilon . \sqrt{2} = 2{\cdot}23$	1·42	86	−14%

The student should calculate for himself the probable errors of the determination when the mean amounts of healing of the groups are greater or less than 2·5 scale divisions.

2. The Increase in Weight of Guinea-pigs due to Vitamin C

Zilva has not published an estimate of the accuracy of his method of determination of vitamin C based on the criterion "increase in weight."

Coward and Kassner (1936) published a curve of response of guinea-pigs given graded doses of vitamin C with a vitamin C-free diet, and also a limited amount of information on the accuracy obtainable in the test as carried out under their own conditions (Chapter V).

From eight groups containing 66 guinea-pigs in all, the standard deviation, σ, of a single observation of the increase in weight of guinea-pigs given daily doses of vitamin C for 6 weeks, was found to be 32·1g. by the method used for calculating the standard deviation from numerous groups of animals in vitamin A tests (Chapter IX). The standard error of the mean of the results from a group of 10 animals was therefore

$\frac{}{\sqrt{10}} - \frac{32{\cdot}1}{\sqrt{10}} = 10{\cdot}2$, and the probable error (1 in 2 chance) was $\frac{2}{3}\epsilon = 6{\cdot}$

The curve of response relating increase in weight of the guinea-pig to dose of vitamin C given (mg. of the International Standard) was found to be expressed by the equation $y = 74{\cdot}3 + 108{\cdot}2 \log. (\log. 10x)$. This is not a simple logarithmic relationship and therefore the percentage error of the test will depend on the particular mean result chosen for the basis of the calculation. The points obtained experimentally for the construction of the curve included a negative one, −37·3g., hence the curve may be used for results showing a certain amount of loss of weight as well as for those showing an increase in weight. This makes it possible to calculate the accuracy of the test for a result in which the doses were large enough to prevent loss in weight, but not large enough to produce an increase in weight, *i.e.* they maintained the weights of the animals. There was no evidence that the variation in response to high doses was different from that in response to low doses.

The accuracy obtainable in determinations by this method has been worked out for results in which (*a*) the doses of Standard and test substance both produced no increase in weight, and (*b*) the doses both produced about 50g. increase in weight, *i.e.* a point somewhat above the middle part of the curve of response. As in other estimates of accuracy, the probable error of the mean result from a group of 10 guinea-pigs was multiplied by $\sqrt{2}$ to obtain the probable error of a comparison in which two groups of 10 guinea-pigs each are used. Thus the probable error of the comparison of two groups of 10 animals each in the experiment of Coward and Kassner was $6{\cdot}8 \times \sqrt{2} = 9{\cdot}62$. The abscissæ corresponding to M, M$\pm$9·62 were found by substituting these values for y in the equation representing the curve of response,

$$y = 74{\cdot}3 + 108{\cdot}2 \log. (\log. 10x).$$

They are set out in Table XXII.

From this table it is evident that the most accurate determinations by this method are made when the doses of test substance and Standard have been so chosen that they have been just sufficient to maintain weight in the guinea-pigs.

Less accurate determinations are made when the doses produce rapid growth.

TABLE XXII

THE ACCURACY OBTAINABLE IN DETERMINATIONS OF VITAMIN C BY THE "INCREASE IN WEIGHT" METHOD, WHEN THE INCREASE IN WEIGHT OF THE GROUPS OF ANIMALS IS (*a*) APPROXIMATELY 0, AND (*b*) APPROXIMATELY 50G.

Mean increase in weight.	Abscissa corresponding to increase in weight.	Abscissa expressed as a percentage of the mean.	Probable error of the determination of vitamin C.
$M = 0$	0·161	100	—
$M+\frac{2}{3}\epsilon.\sqrt{2}=9{\cdot}62$	0·179	111	+11%
$M-\frac{2}{3}\epsilon.\sqrt{2} = -9{\cdot}62$	0·147	91	−9%
$M = 50$	0·395	100	—
$M+\frac{2}{3}\epsilon.\sqrt{2}=59{\cdot}62$	0·539	136	+36%
$M-\frac{2}{3}\epsilon.\sqrt{2}=40{\cdot}38$	0·306	77	−23%

3. REFERENCES

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CHAPTER XII

THE ACCURACY OBTAINABLE IN THE DETERMINATION OF VITAMIN D

THE same procedure is adopted for estimating the accuracy of a vitamin D determination as for any other vitamin. The standard deviation of the response is calculated for the particular test under consideration, or a general estimate of the standard deviation is obtained from the results of a great many tests. Then a curve of response is needed and this may be constructed for each test or a general curve of response may, for certain methods, be constructed by carrying out a special experiment as described in Chapter VI.

I. The " Line " Test

In determinations of vitamin D each litter of rats is divided into two parts as nearly alike as possible. The rats in one half

are given a dose of the Standard; those of the other half are given a dose of the test substance. Thus the animals in a vitamin D test are always paired, and what is required for determining the accuracy of the test is an estimation of the variation in the response of *animals of the same litter* to doses of vitamin D. This can be done in two ways.

A. **Calculation of the standard deviation from pairs of animals of the same litter given the same dose of a substance.**

After a large number of tests has been carried out, all the available results from pairs of animals, both members of which belong to the same litter, and which have received the same dose of the Standard or of test substance, are collected. The two members of any pair should have given equal results. The difference between the results actually given by the two members of a pair is double of the difference of either one from the mean of the two. Therefore all the differences between pairs of results from animals given equal doses of vitamin D can be treated by the formula

$$r=\sqrt{\frac{\Sigma(x_1-x_2)^2}{2N}}$$

where x_1=the result from one member of a pair,

x_2=the result from the other member of a pair,

N=the number of pairs of results.

This is a simpler calculation than the one used for larger groups of animals in each of which all the rats had received the same dose. It should be noted that the same degree of accuracy is obtainable with 100 pairs of animals, each pair from a different family, as is obtainable from 100 animals all of the same family. (The student may work that out for himself.)

Example.—The 25 pairs of results in Table XXIII were taken in order from actual line tests carried out as described in Chapter VI. The results are amounts of healing as judged by the scale depicted in Fig. 28.

TABLE XXIII

THE CALCULATION OF THE STANDARD DEVIATION OF THE RESPONSE OF RATS TO A GIVEN DOSE OF VITAMIN D, FROM 25 PAIRS OF RATS. BOTH RATS OF ANY ONE PAIR RECEIVED THE SAME DOSE OF VITAMIN D, BUT DIFFERENT PAIRS DID NOT NECESSARILY RECEIVE THE SAME DOSE.

Pair.	Amount of healing in pairs of rats.	x_1-x_2.	$(x_1-x_2)^2$.
1	3·5 1·0	2·5	6·25
2	4·5 5·5	−1·0	1·0
3	5·0 5·0	0	0
4	1·0 3·5	−2·5	6·25
5	4·5 4·5	0	0
6	3·5 4·5	−1·0	1·0
7	1·0 2·5	−1·5	2·25
8	2·5 2·5	0	0
9	4·5 3·0	1·5	2·25
10	2·0 3·5	−1·5	2·25
11	5·5 5·0	0·5	0·25
12	4·0 4·0	0	0
13	4·0 4·0	0	0
14	3·5 2·0	1·5	2·25
15	2·0 3·5	−1·5	2·25
16	4·0 3·0	1·0	1·0
17	2·0 3·5	−1·5	2·25
18	5·5 5·0	0·5	0·25
19	5·0 5·0	0	0
20	3·5 4·0	−0·5	0·25
21	2·5 4·0	−1·5	2·25
22	3·0 4·5	−1·5	2·25
23	3·5 2·5	1·0	1·0
24	2·5 2·5	0	0
25	1·0 1·5	−0·5	0·25
			$\Sigma=35{\cdot}50$

Then $$\sigma=\sqrt{\frac{\Sigma(x_1-x_2)^2}{2N}}=\sqrt{\frac{35{\cdot}50}{2\times25}}=\sqrt{0{\cdot}71}=\pm0{\cdot}84$$

Thus the standard deviation of a single determination as calculated from this set of results is $\pm0{\cdot}84$ of a scale division of healing.

B. **Calculation of the standard deviation from the difference between responses of pairs of animals of the same litter, one of each pair having received a dose of Standard and the other a dose of the test substance.**

If there are not enough results available to make a general estimate of the standard deviation as described above, the calculation may be made from all the pairs of animals used in any one test, where one of each pair has been given a dose of the Standard and the other has been given a dose of the test substance. If 10 pairs of animals were used for the determination, 10 differences are available for the calculation. These are put down in one column, and the average of the 10 differences calculated. Then the deviation of each difference from the mean difference is put down, the positive deviation in one column and the negative in another to check the arithmetic by summing. The squares of the deviations are written down in the next column and totalled. The standard deviation of the difference between the response to the Standard and the response to the test substance is then calculated from the formula

$$\sigma=\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

in which x is the difference between results from each pair of rats,

$\bar{x}$ is the mean of the differences,

n is the number of pairs of animals used.

Example.—Ten pairs of rats were used for determining the vitamin D content of a sample of cod liver oil, 1 rat of each pair being given a single dose of 50mg. of the cod liver oil, and the other rat of each pair being given a single dose of 5 units of the International Standard for vitamin D. The line test was carried out in the way described in Chapter VI; the results are summarised in Table XXIV.

TABLE XXIV

THE CALCULATION OF THE STANDARD DEVIATION OF THE DIFFERENCE IN RESPONSE OF 10 PAIRS OF RATS, 1 RAT OF EVERY PAIR HAVING RECEIVED ONE DOSE OF COD LIVER OIL AND THE OTHER RAT OF EVERY PAIR HAVING RECEIVED ONE DOSE OF THE INTERNATIONAL STANDARD.

No. of pair.	Healing from rat given		Difference in healing $(a-b)$, x.	Deviation from mean difference, $x-\bar{x}$.	Square of deviation from mean difference, $(x-\bar{x})^2$.
	(*a*) dose of cod liver oil.	(*b*) dose of Standard.			
1	2·0	2·0	0	−0·25	0·0625
2	2·0	1·0	+1·0	+0·75	0·5625
3	2·0	1·0	+1·0	+0·75	0·5625
4	2·0	1·5	+0·5	+0·25	0·0625
5	1·0	1·0	0	−0·25	0·0625
6	2·5	1·5	+1·0	+0·75	0·5625
7	2·0	2·0	0	−0·25	0·0625
8	2·0	2·0	0	−0·25	0·0625
9	2·0	2·0	0	−0·25	0·0625
10	2·0	3·0	−1·0	−1·25	1·5625
			$\bar{x}=0·25$		$\Sigma=3·6250$

Then $$\sigma^2=\frac{\Sigma(x-\bar{x})^2}{n-1}=\frac{3·6250}{10-1}=0·4028$$

and $$\sigma=0·63$$

Each observation in this method is a difference between a pair of results; it is not the result from one rat. The value of σ so obtained is the standard deviation of the difference between results from pairs of animals, one of which is given a dose of a test substance, and the other a dose of another substance, *e.g.* the Standard. Therefore it does not need to be multiplied by $\sqrt{2}$ when the accuracy of the determination is to be calculated.

A general estimate of the standard deviation may be made from many of these tests by the use of the formula

$$\sigma=\sqrt{\frac{\Sigma d^2}{N-M}}$$

in which Σd^2 is the sum of all the squares of deviations from the various average differences of the tests,

N is the number of pairs of animals,

M is the number of tests.

In 1933, Coward and Key published an estimate of the standard deviation of their vitamin D tests, made by the former of these two methods. It was 0·78, and it was based on 313 pairs of rats, all the pairs from the same litter in which both rats of a pair had received the same dose of any substance, up to the end of August, 1932. Subsequent results obtained in the same laboratory up to the end of June, 1936, have been treated in the same way by Kassner (private communication). The calculation shows a drop from 0·78 to 0·65, the average for the first 6 months of the year 1936. In this period, January to June, 1936, 877 pairs of rats had been used. In the period September, 1932 to June, 1936, 4,183 pairs of rats had been used. To make these estimates of σ into the standard deviation of the difference between two results from a pair of rats, and therefore comparable with the estimates of σ made by the second method, they must be multiplied by $\sqrt{2}$.

For the period September, 1932 to June, 1936, the writer made an estimate of the standard deviation of the vitamin D test by the second method. It included, of course, all the rats which Kassner had used in her calculation. The results of both calculations may be seen in Table XXV.

TABLE XXV

FLUCTUATIONS IN THE STANDARD DEVIATION OF THE DIFFERENCE IN RESPONSE OF PAIRS OF RATS OF THE SAME LITTER TO THE SAME DOSE OF VITAMIN D

Period.	Estimated by:	
	First method.	Second method.
Up to August, 1932	$0·78\times\sqrt{2}=1·10$	not calculated
September–December, 1932	$0·67\times\sqrt{2}=0·95$	0·94
January–December, 1933 ..	$0·73\times\sqrt{2}=1·03$	1·08
January–December, 1934 ..	$0·73\times\sqrt{2}=1·03$	1·00
January–December, 1935 ..	$0·65\times\sqrt{2}=0·92$	0·86
January–June, 1936 ..	$0·65\times\sqrt{2}=0·92$	0·90
September, 1932–June, 1936	$0·69\times\sqrt{2}=0·98$	0·94

The calculation of σ for the period September, 1932 to June, 1936, by the first method was based on 4,183 pairs of rats and the calculation by the second method was based on 4,518 pairs of rats.

The fluctuations in the value of σ from month to month together with the numbers of pairs of rats from which each calculation was made may be seen in Fig. 44. It is of interest

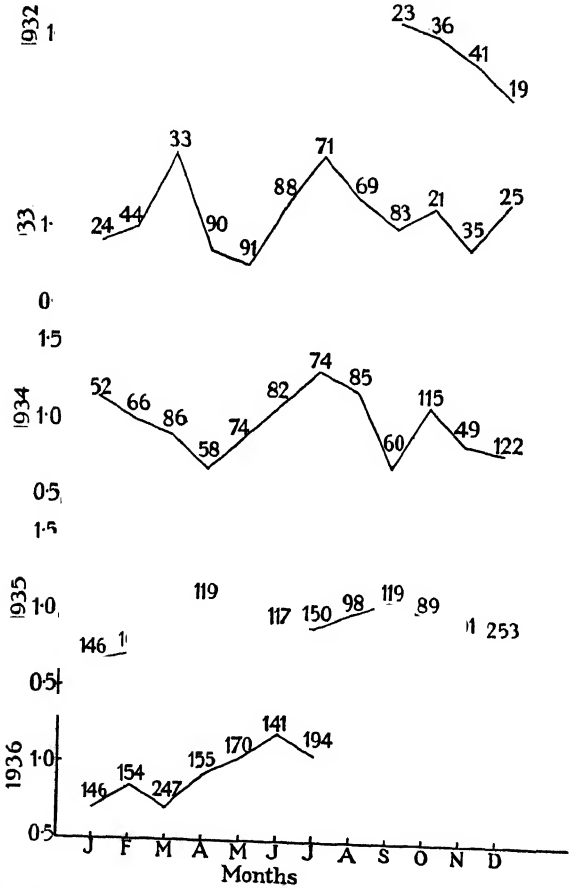

FIG. 44.—Fluctuation in the standard deviation of the difference in response of pairs of rats of the same litter to the same dose of vitamin D. The figures of each month represent the numbers of pairs of rats from which the calculation was made.

because it shows (*a*) that results may gradually become more nearly uniform, and (*b*) that results may be fairly uniform for long periods of time and then, without any apparent reason, become less uniform.

C. **The use of the values obtained by (A) and (B) in estimating the accuracy of a determination.**

As in a vitamin A test, so in a vitamin D test, the accuracy of the determination depends on (*a*) the standard error of the test, and (*b*) the slope of the curve of response relating healing and dose of vitamin D given. If the standard deviation has been determined as described in (A), it must be multiplied by $\sqrt{2}$ to give the standard deviation of the difference between results from pairs of animals. Values obtained from experiments in the writer's laboratory have been used in the following calculation to demonstrate the method of estimating the accuracy of a determination of vitamin D.

The standard deviation, σ, of the difference in healing of two members of a pair of rats, one of which has been given a dose of test substance and the other a dose of the Standard of reference, was found to be 0·94. Then the standard error, ϵ, of the average difference between 10 pairs of rats similarly treated is $\frac{\sigma}{\sqrt{10}}=0{\cdot}30$, and the probable error, $\frac{2}{3}.\epsilon$, is 0·20. Then, as explained for vitamin A, the inaccuracy of the result cannot be calculated directly as the ratio between $M-\frac{2}{3}\epsilon$, M and $M+\frac{2}{3}\epsilon$ for the curve of response relating healing and dose of vitamin D given is not a straight line but a logarithmic curve. The inaccuracy of the result must be determined from the abscissæ corresponding to these values in the curve of response. The curve of response used in the writer's laboratory was of the form $y=4{\cdot}7+3{\cdot}1$ log. x throughout the range 1 to about 4·5 divisions of y, where y=the amount of healing, six stages being depicted though intermediate stages were distinguishable, and x the dose in units of vitamin D given. Below 1·0 the shape was not determined; from 4·5 to 6 it flattened somewhat, which probably indicates that the stages of healing, called 5 and 6, were not well chosen. If stage 5 had been called 6 and stage 6 had been called 7 the curve relating healing and the logarithm of the dose of vitamin D given would have been a straight line throughout its length. The upper part of the curve is, however, not used, as in biological assays generally, the investigator experiments with different doses until he obtains results lying more nearly in the middle part of the curve of response, and it is from these he makes his determination.

The abscissæ of the curve $y=4{\cdot}7+3{\cdot}1$ log. x corresponding to the ordinates $M-\frac{2}{3}\epsilon$, M and $M+\frac{2}{3}\epsilon$ may be determined directly from the curve or by substituting their values for y in the equation of the curve. If M is supposed to have the value 2·5, then the error of a determination is worked out as in Table XXVI.

TABLE XXVI

THE CALCULATION OF THE PROBABLE ERROR OF A DETERMINATION OF VITAMIN D WHEN 10 PAIRS OF RATS ARE USED, ONE RAT OF EACH PAIR BEING GIVEN A DOSE OF THE STANDARD AND THE OTHER A DOSE OF THE TEST SUBSTANCE.

Average healing.	Abscissa corresponding to average healing.	Abscissæ expressed as a percentage of the mean.	Probable error of the determination of vitamin D.
$M = 2{\cdot}50$	0·195	100	—
$M+\frac{2}{3}\epsilon = 2{\cdot}70$	0·226	116	+16%
$M-\frac{2}{3}\epsilon = 2{\cdot}30$	0·168	86	−14%

The accuracy of the determination can be worked out more directly as follows: The difference between two abscissæ corresponding to a difference of one scale division of healing is determined from the equation $y=4{\cdot}7+3{\cdot}1x$ where x is the logarithm of the dose, and 3·1 is the tangent of the angle that the curve makes with the axis of X, *i.e.*

$$\frac{\text{an increment of Y}}{\text{the corresponding increment of X}}=3{\cdot}1$$

Therefore an increment of 1 for Y corresponds to an increment of $\frac{1}{3{\cdot}1}$ for X=0·32. Thus an increment of one scale division of healing corresponds to multiplying the dose by the antilog. of 0·32, *i.e.* by 2·09. This almost corresponds to a simple doubling of the dose for each increment of a scale division of healing.

Now the probable error of an estimation, $\frac{2}{3}\epsilon=0{\cdot}20$, corresponds to an increment of $\frac{0{\cdot}20}{3{\cdot}1}$ for $X=\pm 0{\cdot}0645$, which is the log. of 1·16 or 0·86. There is a 1 in 2 chance that the result

may be outside 1·16 or 0·86 times the true result; *i.e.* it may be more than 116 or less than 86% of the true result.

This method of calculation is obviously only applicable to logarithmic curves or curves which are logarithmic throughout the greater part of their length.

For the calculation of the error of a result in a test for which the curve of response is not a simple logarithmic one, the calculation of the error of vitamin C determinations may be consulted.

At this stage the student is advised to work out the limits (as percentages of the true value) beyond which a result will lie 1 in 22 times (for which he will use 2ϵ) and also the limits beyond which a result will lie 1 in 100 times (for which he will use $2{\cdot}576\epsilon$). It is also very instructive to work out all of these limits for different tests in which different numbers of pairs of animals (say, 1, 2, 4, 9, 16, 25, 36, 100 pairs) are supposed to have been used. The results should be plotted on graph paper. The student will then be able to decide for himself how far it is worth while increasing the number of animals in a test in order to increase the degree of accuracy of his results. (The graphs obtained by plotting these results are the best test of the accuracy of one's arithmetic. They should, of course, be perfectly smooth curves.)

Morgan (1932) used a method of measuring the healing in the line test which appeared to reduce the error of his assays made on 10 pairs of rats to +7·7 or −7·0%. He made camera lucida drawings of the sections of bones (constant magnification) and measured the areas of calcification by a planimeter, assigning only half the values found to the areas of scattered calcification. This estimate was based on only 200 rats, and the period covered was only 7 months. Hence it was not possible to say whether the low error was due to a more accurate method of measuring healing or whether the 7 months was a period of less inaccuracy such as occurs from time to time, unaccountably, in most laboratories.

D. The accuracy of a determination when neither a general estimate of the standard deviation nor a general curve of response has been made previously.

Suppose the following results had been obtained in a "line" test in which doses of 5 and 10 units of the Standard

and 50mg. of cod liver oil respectively had been given to three groups of rats, each litter of rats being equally represented in each of the three groups.

	Healing of rats given :		
	5 units Standard.	10 units Standard.	50mg. cod liver oil.
Litter 1 ..	1·5	2·5	1·5
	1·0	3·0	2·0
	2·0	2·5	1·5
,, 2 ..	0·5	2·0	1·5
,, 3 ..	2·0	3·5	2·5
,, 4 ..	1·5	2·0	2·0
	1·0	2·0	1·5
	1·0	1·5	1·5
,, 5 ..	1·5	3·0	2·5
	2·0	3·5	2·0
Average	1·40	2·55	1·85

Five units of the Standard produced an average healing of 1·40. Ten units of the Standard produced an average healing of 2·55. The two average healings are plotted against the logs. of the doses of vitamin D given and a straight line drawn between the points so obtained. The abscissa of this curve corresponding to 1·85 (the average healing brought about by 50mg. cod liver oil) is found to be 0·817. Therefore 50mg. cod liver oil contain 6·56 units (the antilog. of 0·817) of vitamin D. Therefore the oil contains 130 units of vitamin D per gram.

The accuracy of the test is worked out as follows: The standard deviation of the difference between pairs of rats (one of which was given a dose of Standard and the other a dose of oil) is first determined. Since the rats were used in threes, two differences are obtained from each three, the difference in healing between the rat given 50mg. cod liver oil and that given 5 units of the Standard, and the difference in healing between the rat given 50mg. cod liver oil and that given 10 units of the Standard. This involves using each rat given 50mg. cod liver oil twice in the calculation, but this is allowable since it is a calculation of differences between results which is being made, not a calculation directly from results (Table XXVII).

TABLE XXVII

CALCULATION OF THE STANDARD DEVIATION OF THE DIFFERENCE IN RESULTS FROM PAIRS OF RATS, ONE OF EACH PAIR HAVING BEEN GIVEN A DOSE OF THE STANDARD AND THE OTHER A DOSE OF COD LIVER OIL.

5 units Standard.	50mg. cod liver oil.	Difference between responses, x.	Deviation from mean, $x-\bar{x}$.	Square of deviation from mean, $(x-\bar{x})^2$.
1·5	1·5	0	−0·45	0·2025
1·0	2·0	1·0	+0·55	0·3025
2·0	1·5	−0·5	−0·95	0·9025
0·5	1·5	1·0	+0·55	0·3025
2·0	2·5	0·5	+0·05	0·0025
1·5	2·0	0·5	+0·05	0·0025
1·0	1·5	0·5	+0·05	0·0025
1·0	1·5	0·5	+0·05	0·0025
1·5	2·5	1·0	+0·55	0·3025
2·0	2·0	0	−0·45	0·2025
		4·5	0	2·2300
		$\bar{x}=0·45$		

r of this set of pairs of results $=\sqrt{\frac{2·2300}{10-1}}=\sqrt{0·2478}=0·498$

50mg. cod liver oil.	10 units Standard.			
1·5	2·5	1·0	+0·3	0·09
2·0	3·0	1·0	+0·3	0·09
1·5	2·5	1·0	+0·3	0·09
1·5	2·0	0·5	−0·2	0·04
2·5	3·5	1·0	+0·3	0·09
2·0	2·0	0	−0·7	0·49
1·5	2·0	0·5	−0·2	0·04
1·5	1·5	0	−0·7	0·49
2·5	3·0	0·5	−0·2	0·04
2·0	3·5	1·5	+0·8	0·64
		7·0	0	2·10
		$\bar{x}=0·7$		

σ of this set of pairs of results $=\sqrt{\frac{2·10}{10-1}}=\sqrt{0·23\dot{3}}=0·483$

The standard deviation judged from the whole of the results is

$$\sqrt{\frac{2·23+2·10}{9+9}}=\sqrt{\frac{4·33}{18}}=\sqrt{0·2406}=0·491$$

Then, since there were twenty differences from which to calculate the value of σ, the standard error of the whole test is

$$\epsilon=\frac{\sigma}{\sqrt{n}}=\frac{0{\cdot}491}{\sqrt{20}}=\pm 0{\cdot}110$$

Now, since a difference in healing of 1·15 (=2·55−1·40) is obtained by doubling the dose of vitamin D given (10 units and 5 units), *i.e.* a difference in healing of 1·15 corresponds to a difference of 0·30103 (log. 2) in the abscissæ of the curve of response, a difference of $\pm 0{\cdot}110$ ($=\epsilon$) corresponds to a difference of

$$\pm\frac{0{\cdot}30103\times 0{\cdot}110}{1{\cdot}15}=\pm 0{\cdot}0288$$

$$+0{\cdot}0288=\text{log. } 1{\cdot}0686$$

and $$-0{\cdot}0288=\bar{1}{\cdot}9712=\text{log. } 0{\cdot}93584$$

There is, therefore, 1 chance in 3 that the determination, 130 units per gram, may be at least 1·07 or 0·94 times the true result, *i.e.* that it is 7% too large or 6% too small.

By making similar calculations with 2ϵ instead of ϵ, the limits beyond which there would be a 1 in 22 chance of the result lying, would be found. With $2{\cdot}576\epsilon$, the limits for a 1 in 100 chance would be found.

E. The correlation coefficient as a means of determining whether (a) initial weight, or (b) sex, has any influence on the amount of healing brought about by a given dose of vitamin D.

Sometimes in vitamin determinations it is desired to discover whether some particular factor in the test has any influence on the result. Provided all other factors are constant, or as nearly constant as the worker can make them, the possibility of a correlation between the one factor and the result may be calculated by the formula :

$$r=\frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2.\,\Sigma(y-\bar{y})^2}}$$

in which r=the correlation coefficient,

$(x-\bar{x})$ is the difference of each observation x from the mean $\bar{x}$,

$(y-\bar{y})$ is the difference between the corresponding value of y and the mean $\bar{y}$.

The greater the value of r, the greater is the probability that a correlation exists between the two sets of observations, and

the smaller the number of pairs of observations from which a given value of r is obtained the greater is the probability that a correlation exists between the two sets of observations; *e.g.* if 10 pairs of observations are available for the calculation and r is found to be 0·70, there would be about a 2·4% chance, only (P=0·024), that the apparent correlation was due to random sampling, *i.e.* 1 chance in 40 that there was no correlation between the sets of observations, from which it would be fairly safe to assume that the two observations *were* correlated. Other probabilities for different numbers of pairs of observations are obtained from the appropriate tables, *e.g.* Table VA, p. 212, of the 6th Edition of Fisher's *Statistical Methods for Research Workers*.

A diagram on graph paper, in which one variable of each pair of observations is plotted against the other variable of the pair giving a " dot diagram," will often indicate a possible correlation which is worth following up.

Coward and Key (1933) found by calculation that the initial weight of the rat (*i.e.* its weight when it was first given the rachitogenic diet) was correlated with the amount of healing produced by a dose of vitamin D. The figures for male and female rats were calculated separately lest sex should also have an influence. The figures obtained are summarised in Table XXVIII.

TABLE XXVIII

CORRELATION BETWEEN THE INITIAL WEIGHT OF THE RAT (*i.e.* WHEN FIRST GIVEN THE RACHITOGENIC DIET) AND THE AMOUNT OF HEALING BROUGHT ABOUT BY A GIVEN DOSE OF VITAMIN D.

Daily dose.	No. of rats.	Value of r.	P=probability that there is no correlation.
0·5 unit D	54♂ 41♀	0·3388 0·2557	0·012 very slightly >0·1
0·25 unit D	128♂ 133♀	0·2286 0·2780	0·01 <0·01

Thus, in three of the four groups, there was very good evidence of the influence of initial weight on the amount of healing produced by a given dose of vitamin D. In the other group the evidence was less good, but in that group there were only 41 rats.

A comparison was made of the healing brought about by the same dose of vitamin D in pairs of rats in which one was a male and one a female and the initial weights of the two in any pair did not differ by more than 2g. (thus eliminating influence of initial weight). The average healing of the males in 72 such pairs was 2·96 and of the females 2·79. The difference was not significant. Therefore it was concluded that, when differences in initial weight were eliminated, the sex of the animals had no influence on the amount of healing brought about by a given dose of vitamin D.

2. The "X-ray" Method

There is no essential difference between the "line test" and the "X-ray" methods of determining vitamin D, except that in the former the healing is judged from the cut section of a bone and in the latter it is judged from an X-ray photograph of the same structure. The method of estimating the accuracy obtainable by the two methods is, therefore, the same.

Bourdillon, Bruce, Fischmann and Webster (1931) chose 14 days for the preparatory period for their animals and 14 days as the curative period, and worked out the accuracy which they had obtained by the use of this method. They constructed a scale of healing consisting of twelve divisions ranging from 0 for no healing to 12 for complete healing. Their curve of response was logarithmic throughout the range of scale numbers 3–9. Two scale divisions corresponded to a doubling of the dose of vitamin D given, hence the curve was of the shape $y=a+b$ log. x (actually $2{\cdot}88+6{\cdot}1$ log. x as determined from their published graph). The standard deviation of the difference between the healing from the two members of a single pair of rats in their experiments was found to be 1·5288 of their scale divisions. Thus the standard error for 10 pairs of rats was $\epsilon=\frac{1{\cdot}5288}{\sqrt{10}}=\pm 0{\cdot}4735$ and the probable error (1 in 2 tests) was $\frac{2}{3}\epsilon=\pm 0{\cdot}3156$. The accuracy of their determination can then be worked out by the same methods as are used for working out the accuracy of the line tests, *i.e.* (*a*) directly from the curve of response, reading values for M, and $M\pm\frac{2}{3}\epsilon$ from the graph of the curve, or by substituting in the equation which is approximately $y=2{\cdot}9+6{\cdot}1$ log. x,

or (*b*) from the difference between abscissæ corresponding to a range of two scale divisions of healing calculating the difference in abscissæ corresponding to a difference of $\pm\frac{2}{3}\epsilon$ from which the percentage error of the test is determined.

The student is advised to work this out for himself, not only for the probable error (1 in 2 tests) for which $\frac{2}{3}\epsilon$ is used, but also for chance of 1 in 22 for which 2ϵ is used and 1 in 100 for which $2{\cdot}576\epsilon$ is used. It can also be worked out for different numbers of pairs of rats.

3. The "Ash Content of the Bones" Method

It was shown in Chapter VI that determinations of vitamin D by this method involved the testing of three doses of the Standard and three doses of the substance under examination in the hope of finding at least one dose of the test substance giving a result equal to at least one given by one dose of the Standard. Each group of rats was to contain the same number of animals from each litter used. The doses chosen for test were to be in the ratio 4 : 2 : 1 for both Standard and test substance. Thus there would be six groups of animals from which to calculate the accuracy of the determination.

The average standard deviation could be calculated from the six groups, but this would not take into account the fact that litters had been distributed equally in the six groups. As this precaution gives an increased accuracy to the test, it must be taken into account in assessing the accuracy. Therefore it is preferable to estimate the standard deviation of the differences between results from pairs of animals. The largest amount of information will be obtained by taking pairs of animals in which one of the pair has received a dose of vitamin D known to be half of the dose received by the other rat of the pair. When 3 rats have received doses of the ratio 4 : 2 : 1 two pairs of results may be taken for the purpose of estimating the standard deviation, *i.e.* the result from the rats receiving the doses in the ratio 4 : 2 and those from the rats receiving the doses in the ratio 2 : 1. Thus, although one rat seems to appear twice, no *difference* appears twice which is the value to be measured. The standard deviation of these differences from the mean difference is then calculated for each group of rats and the general value obtained in the usual way.

Example.—The figures obtained in the determination described in Chapter VI are used here to demonstrate the method of estimating the standard deviation in this type of experiment. In Table XXIX have been arranged in the first two columns (1 and 2) the numbers of the rats whose values for the ash content of their bones have been arranged in the next two columns (3 and 4). The differences (x) in ash content between the respective pairs of rats have been arranged in the next column (5). These differences have been totalled and averaged ($\bar{x}$), and in column 6 are the deviations ($x-\bar{x}$) of each difference (x) from the mean difference ($\bar{x}$). In column 7 the deviations are squared and totalled. The value for σ is then determined by the usual formula

$$\sigma=\sqrt{\frac{\Sigma(x-\bar{x})}{n-1}}$$

In this experiment $\sigma=\pm 3{\cdot}98$, which means that one-third of these differences lie outside the mean difference $\pm 3{\cdot}98$, *i.e.* outside $8{\cdot}28\pm 3{\cdot}98$, *i.e.* outside the range 4·30–12·26. Actually seven of the twenty-four differences lie outside this range, which is very close to the theoretical eight considering the small size of the sample (twenty-four differences or observations). Similarly, one-half of the observations should lie outside the mean $\pm\frac{2}{3}\sigma$, *i.e.* outside $8{\cdot}28\pm 2{\cdot}66$, *i.e.* outside the range 5·62 to 10·94. Actually eleven of the twenty-four observations lie outside this range. Also 4·5% (one out of twenty-two) of the observations should lie outside the mean $\pm 2\sigma$, *i.e.* outside $8{\cdot}28\pm 2\times 3{\cdot}98$, *i.e.* outside $8{\cdot}28\pm 7{\cdot}96$, *i.e.* outside the range 0·32 to 16·24. Actually two out of the twenty-four do so.

The standard error, ϵ, of the mean of the differences between the results from these 24 pairs of rats, one of which received half the dose of vitamin D that the other received, is given by

$$\epsilon=\frac{\sigma}{\sqrt{24}}=\pm\frac{3{\cdot}98}{4{\cdot}9}=\pm 0{\cdot}81$$

Therefore there is a 1 in 2 chance of an error of $\frac{2}{3}\epsilon=\pm 0{\cdot}54$, a 1 in 3 chance of an error of $\epsilon=\pm 0{\cdot}81$ and a 1 in 22 chance (4·5%) of an error of $2\epsilon=\pm 1{\cdot}62$ in the difference between the percentage ash produced by one dose of vitamin D and that produced by half the dose, in this particular experiment when 36 rats were used.

TABLE XXIX

CALCULATION OF THE STANDARD DEVIATION (σ) OF THE DIFFERENCE BETWEEN THE ASH CONTENT (EXPRESSED AS A PERCENTAGE OF THE FAT-FREE DRY BONE) OF BONES OF PAIRS OF RATS, ONE OF WHICH HAD RECEIVED HALF AS MUCH VITAMIN D AS THE OTHER.

	1	2	3	4	5	6	7
	Rat number		Percentage ash of rat		Difference, (x).	Deviation from mean, ($x-\bar{x}$).	Square of deviation from mean, $(x-\bar{x})^2$.
	on dose D.	on dose D/2.	on dose D.	on dose D/2.			
Cod Liver Oil	185	182	43·00	35·25	7·75	−0·53	0·2809
	182	181	35·25	24·90	10·35	+2·07	4·2849
	238	235	46·04	36·39	9·65	+1·37	1·8769
	235	237	36·39	32·23	4·16	−4·12	16·9744
	294	296	47·26	36·14	11·12	+2·84	8·0656
	296	298	36·14	33·71	2·43	−5·85	34·2225
	347	351	46·25	37·89	8·36	+0·08	0·0064
	351	350	37·89	31·43	6·46	−1·82	3·3124
	256	257	48·75	35·71	13·04	+4·76	22·6576
	257	254	35·71	24·54	11·17	+2·89	8·3521
	368	369	43·83	37·74	6·09	−2·19	4·7961
	369	373	37·74	34·16	3·58	−4·70	22·0900
International Standard for Vitamin D	186	183	35·51	37·59	−2·08	−10·36	107·3296
	183	184	37·59	20·64	16·95	+8·67	75·1689
	240	239	41·34	30·48	10·86	+2·58	6·6564
	239	236	30·48	25·14	5·34	−2·84	8·0656
	295	297	41·86	32·90	8·96	+0·68	0·4624
	297	299	32·90	23·99	8·91	+0·63	0·3969
	348	346	42·79	32·25	10·54	+2·26	5·1076
	346	349	32·25	23·04	9·21	+0·93	0·8649
	258	259	42·18	29·54	12·64	+4·36	19·0096
	259	255	29·54	24·70	4·84	−3·44	11·8336
	371	370	41·08	32·36	8·72	+0·44	0·1936
	370	372	32·36	22·76	9·60	+1·32	1·7424
				Total ..	198·65	+35·88	363·7513
				Mean=$\bar{x}$=8·28		−35·85	
						0·03	

Then $$\sigma^2=\frac{\Sigma(x-\bar{x})^2}{n-1}=\frac{363{\cdot}7513}{24-1}=15{\cdot}8513$$

and $$\sigma=\sqrt{15{\cdot}8513}=3{\cdot}98$$

That is, the standard deviation of the difference between the ash content of the bones of 2 rats, one of which had received half as much vitamin D as the other, is 3·98 as]
of fat-free dry bone.

A question of principle in biological assay may conveniently be discussed here in connection with this test. Rat 183 did not grow as much as the rest during the first 4 weeks of the test (only 7g. against an average of 24 for the others), and in the last 2 weeks lost 9g. in weight. This was not due to lack of food as evidenced by daily records of amount of food eaten. As there is evidence that spontaneous healing of rickets may take place in a rat which for any reason loses weight during the test, there is a temptation to discard the result from rat 183 entirely. Here arises the question: When is it permissible to discard a result? Of course, if a result is spoilt by a known mistake in manipulation such as spilling the ash before weighing, then the result must be discarded. If, however, it is merely thought that the apparently abnormal result *must* be due to some abnormal behaviour of the rat, then the only safe procedure is to include that result in the rest. It can never be certain that the abnormality is not really one of the extreme results occurring in every normally distributed set of observations. Indeed, it may do much to counterbalance the rest of the results if they have swung a little too far in the opposite direction. Also, if an extreme result is discarded once (for what may appear to be a very good reason), it may be a temptation to discard one which is not nearly so extreme. It would be difficult to know where to draw the line. The safe rule, then, is "never discard a biological result." It would be an instructive exercise for the student to work out the results in Table XXIX, omitting rat 183, to see how far the result would be affected by discarding it.

Like so many biological responses, the response of rats to doses of vitamin D, as measured by the ash content of their bones, bears a logarithmic relationship to the dose of vitamin D given. No one has ever attempted to construct a curve of response relating percentage of ash in bones to dose of vitamin D given that could be used for comparing results obtained by any but a test carried out at the same time and with rats from the same litters as those used in constructing the curve. Hume, Pickersgill and Gaffikin (1932) have shown how impossible it would be to do this. They constructed several curves of response relating percentage of ash in the bone to the dose of vitamin D given. For each curve several litters of rats were divided into groups, each group containing equal numbers of

rats from all the litters used. The different groups were given graded doses of vitamin D as irradiated ergosterol in olive oil, all the rats of any one group being given the same dose. When the average ash contents of the groups of each experiment were plotted against the logs. of the doses (as International units) of vitamin D given, the different experiments made curves of different slopes, starting from different points but sloping towards a point common to them all at a dosage of about 5 units (Fig. 27, Chapter VI).

The impossibility of constructing one curve of response for interpreting all comparisons to be made subsequently is due to the fact that it is impossible to ensure the same ash content in the bones of the "No dose" rats in different experiments carried out at different times. Hence the only way to use the "ash content of the bones" method is that already described in which three doses of the Standard in the ratio 4 : 2 : 1 are given and three doses of the test substance in a similar ratio. This procedure gives two curves of response in the one experiment. If they are found to be superposable, *i.e.* if they have the same slope, the calculation of the result is simple and also the calculation of the error. If the two sets of results give curves of different slopes, the slopes must be averaged for assessing the error.

Example.—In the experiment which has already been described as an example of this method of determining vitamin D (Chapter VI, p. 122), and whose standard deviation of the mean difference between results from rats given doses and half those doses of vitamin D has been calculated above, the curves of response of the groups given different doses of Standard and different doses of cod liver oil respectively, were

for the Standard, $y=69{\cdot}85+28{\cdot}92 \log. x$
for the cod liver oil, $y=47{\cdot}89+26{\cdot}08 \log. x$

The slopes of these curves are nearly the same (28·92, 26·08). They are averaged for the purpose of assessing the error of the test : $b=\frac{1}{2}(28{\cdot}92+26{\cdot}08)=27{\cdot}50$.

Then the probable error of this particular determination, in which 24 pairs of results were available for the calculation, is calculated as follows :

In a curve whose equation is $y=a+27{\cdot}50x$

$$\frac{\text{an increment of Y}}{\text{the corresponding increment of X}}=\frac{27{\cdot}50}{1}$$

The probable error of the difference between results from 24 pairs of rats one of which had been given only half as much vitamin D as the other, was found to be 0·54.

Therefore $\frac{\text{an increment of 0·54 in Y}}{\text{the corresponding increment of X}} = \frac{27·50}{1}$

Therefore the corresponding increment of

$$X = \frac{0·54}{27·50} = 0·0196 = \text{log. } 1·0461$$

Therefore this determination may be 1·046 or 0·956 times the true determination, *i.e.* it may be 4·6% too great or 4·4% too small (probable error 1 chance in 2). There is also 1 chance in 22 that the determination is 14·5% too great or 12·7% too small.

It must be realised that this is not a general estimate of the accuracy of determinations by the " ash content of the bones " method. It is an estimate of the accuracy obtained in one particular determination in which 36 rats were used, and fairly steep curves of response were obtained.

To compare the accuracy obtainable in this test with that obtainable in other tests, the standard error using 10 pairs of rats should be calculated and an average made of the slopes of all the curves of response available.

4. The " Increase in Weight " Method

Coward, Key and Morgan (1932) worked out the accuracy obtainable in the determination of vitamin D by the " increase in weight " method from the very limited data available. The calculation was made in the same way as that for the accuracy of the determination of vitamin A by the " increase in weight " method. The calculation was as follows:

The curves of response relating the dose of vitamin D given and the increase in weight in 5 weeks of rats which had become steady in weight on a shortage of vitamin D, and were then given doses of vitamin D, were found to be:

Bucks .. .. .. $y = 92·82 + 30·02 \log. x$
Does .. .. $y = 63·14 + 22·74 \log. x$

in which y = the increase in weight in 5 weeks, and x = the daily dose of vitamin D in International units.

The standard deviation, σ, of a single determination was found to be :

Bucks	15·80
Does	8·76

Thus the standard deviation of the logarithm (to the base 10) of the result is :

Bucks $\frac{15\cdot80}{30\cdot02}=0\cdot526=\lambda_1$

Does $\frac{8\cdot76}{22\cdot74}=0\cdot385=\lambda_2$

Then the curve of response of groups of equal numbers of bucks and does would have a slope of $\frac{1}{2}(30\cdot02+22\cdot74)=26\cdot38$ and the standard deviation of such groups would be

$$\tfrac{1}{2}(15\cdot80+8\cdot76)=12\cdot28$$

The accuracy obtainable in the test is then worked out either from the curve drawn on graph paper, or by substituting values for M, $M\pm\frac{2}{3}$ in the equation for the curve, or by the method of determining the increment of x corresponding to the increments $\pm\frac{2}{3}\epsilon$ in y, using in each method $\sqrt{2}.\epsilon$ instead of ϵ for the error of a determination of the vitamin D potency of one substance made with a simultaneous test of the Standard of reference. The probable error of a determination of vitamin D in which 10 animals (5 males and 5 females) are used for the test substance and 10 similar animals for the Standard is +37·6 or −27·3%. This is very much higher than the error of the determination of vitamin D by any of the other methods.

5. References

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APPENDIX I

SECOND CONFERENCE ON VITAMIN STANDARDISATION

held in London from June 12th to 14th, 1934

Preliminary Note

An International Vitamin Conference, attended by most of the experts responsible for the present report, was held in London in June, 1931, under the auspices of the Permanent Commission on Biological Standardisation of the League of Nations Health Organisation. The report of the earlier Conference,* published in 1931, recommended for International adoption standards and units for vitamins A, B_1, C and D, which were to be "provisional for two years." Since certain of the standard preparations recommended for adoption were not available for general use until 1932, the second Conference was postponed until 1934, at which date two years' experience of the practical application of the provisional standards was available.

The following report should therefore be regarded as a revision of the report of the 1931 Conference. In making its decisions, the present Conference had also at its disposal the results of many important new investigations connected with vitamin standardisation. It is to be noted that, in this report, the standards and units recommended are no longer termed "provisional." While the advance of scientific knowledge may at some future date make changes in the adopted standards and units desirable, it was not felt necessary, as before, to assign provisional periods for their use.

The time may shortly arrive when the cumbrous method of comparing the vitamin activity of test materials with the activity of a chosen standard will be obsolete, and vitamin potency will be expressed in terms of the exact amount of pure active vitamin present. The Conference, however, considered it desirable that, for the present, vitamin potency should, in the case of all the vitamins dealt with in its report, continue to be estimated by means of biological tests in comparison with a chosen standard, even in those cases in which pure substances possessing high vitamin potency have been isolated, thus making it possible to estimate with considerable accuracy, by chemical and physical methods, the amount of these substances present in materials under test.

* *Report of the Conference on Vitamin Standards.* Publications Department of the League of Nations No. C.H. 1055 (1). Geneva, 1931.

As in the 1931 Report, standards and units are recommended for only four of the known vitamins—namely, vitamins A, B_1, C and D. Consideration was given to the possibility of adopting standards for vitamins B_2 and E, but it was felt that our knowledge of the nature and possible complexity of these vitamins, and of the pathological results to which their absence gives rise, is still insufficient to justify the adoption of standards and units for them.

The standards for vitamins A and C have been altered. Those chosen for these vitamins in 1931 were found to have certain defects which impaired their usefulness; the substitution of more clearly defined and more easily reproducible chemical substances is a definite step in advance. No change has been recommended in the standards for the vitamins B_1 and D. The former has proved highly convenient in practice—of all the standards chosen by the 1931 Conference the vitamin B_1 standard has perhaps proved most satisfactory—and a large stock, sufficient to last for many years, is available at the central distributing institution, the National Institute for Medical Research, London. In view of all the circumstances, its replacement by a purer vitamin B_1 preparation does not seem at present desirable. The vitamin D standard also remains unaltered, with the proviso that it may be replaced when exhausted (or if it should become for any reason unsatisfactory) by crystalline vitamin D in suitable solution. Large quantities of the original standard solution of irradiated ergosterol are still available. Certain recently observed anomalies, in the antirachitic action on certain species, of different substances containing equal numbers of International vitamin D units, to which brief reference is made in the report, suggest that a re-examination of the suitability of the present vitamin D standard may become necessary at some future date.

The units of vitamin activity remain the same in all cases. Where a change has been made in the standard material, the old units have been re-stated in terms of the newly chosen substance. The desirability of leaving the original units unaltered is emphasised by the fact that certain of the units recommended by the 1931 Conference have been adopted in the pharmacopœias of certain countries.

The majority of the members of the Conference have in recent years carried out experiments bearing directly on problems of vitamin standardisation, the results of which proved to be of the greatest value to the Conference. In addition to the members of the Conference, many workers in different countries, whose names do not appear in the Report, helped to carry out the recommendations for research made by the 1931 Conference, and placed valuable information at the disposal of the present meeting. Thanks are due to such workers, without whose active and untiring co-operation the Conference could not have undertaken the task set before it.

REPORT OF THE CONFERENCE

List of Participants

Chairman : Dr. E. MELLANBY, Secretary, Medical Research Council of Great Britain, London.

Professor J. C. DRUMMOND, Professor of Biochemistry, University College, London.

Professor H. v. EULER, Professor of Biochemistry, University of Stockholm.

Professor L. J. FRIDERICIA, Director, Institute of Hygiene, University of Copenhagen.

Professor B. C. P. JANSEN, Professor of Physiological Chemistry, University of Amsterdam.

Professor P. DE MATTEI, Director, Institute of Pharmacology, University of Pavia.

Dr. E. M. NELSON, Senior Chemist, Bureau of Chemistry and Soils, U.S. Department of Agriculture, Washington, D.C.

Professor E. POULSSON, Director, State Vitamin Institute, Oslo.

Mme. RANDOIN, Directeur du laboratoire de physiologie de la nutrition à l'École des Hautes-Études, et Ministère de l'Agriculture, Paris.

Professor H. STEENBOCK, Professor of Agricultural Chemistry, University of Wisconsin, Madison, U.S.A.

Professor A. SZENT-GYÖRGYI, Institute of Medical Chemistry, University of Szeged, Hungary.

Dr. H. CHICK, Lister Institute of Preventive Medicine, London. } Technical Secretaries

Dr. W. R. AYKROYD, Health Section, League of Nations Secretariat, Geneva. } Technical Secretaries

The following also were present :

Dr. M. H. BROWN, Connaught Laboratories, Toronto, Canada.

Dr. K. H. COWARD, Laboratory of the Pharmaceutical Society of Great Britain, London.

Sir Henry H. DALE, Director, National Institute for Medical Research, London.

Dr. P. HARTLEY, National Institute for Medical Research, London.

Miss E. M. HUME, Lister Institute of Preventive Medicine, London.

Dr. A. JUNG, Institute of Physiological Chemistry, University of Basle.

Dr. Ch. LORMAND, Directeur du Laboratoire national de contrôle des médicaments, Paris.

Dr. R. MENDEZ, Technical Institute of Pharmacy and Biology, Madrid.

Dr. R. A. MORTON, Department of Chemistry, University of Liverpool.

Dr. R. A. PETERS, Professor of Biochemistry, University of Oxford.

Dr. O. ROSENHEIM, National Institute for Medical Research, London.

Dr. M. TSURUMI, Member of the League of Nations Health Committee (Japan).

Mr. T. A. WEBSTER, National Institute for Medical Research, London.

Dr. S. S. ZILVA, Lister Institute of Preventive Medicine, London.

The meeting was opened by Sir George BUCHANAN, who welcomed the members on behalf of the Health Committee of the League of

Nations, after which Sir Henry H. Dale gave a brief review of the present position with regard to International vitamin standardisation.

I. Vitamin A

(a) *International Standard*

The Conference recommends that pure β-carotene be adopted as the International Standard for vitamin A. The Standard Preparation shall conform to the requirements stated in Note 1 in regard to its chemical and physical constants.

(b) *Definition of Unit*

The Conference recommends that the unit for vitamin A provisionally adopted at the 1931 Conference shall be maintained. It has been established that one such unit is contained in 0·6 microgram (0·6γ) of pure β-carotene.

The International unit for vitamin A recommended for adoption shall be defined as the vitamin A activity of 0·6 microgram (0·6γ) of the International Standard Preparation.

Daily doses of 2–4 International units of vitamin A, when administered to young rats suitably prepared on a vitamin A-deficient diet, have been found adequate to restore growth; somewhat larger doses are required for the cure of xerophthalmia.

(c) *Mode of Preparation*

It is recommended that the Health Organisation of the League of Nations shall be requested to obtain a sample of β-carotene as defined by the Conference (see Note 1), and that the National Institute for Medical Research, London, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations,* shall undertake the care, storage and distribution of the International Vitamin A Standard so obtained.

(d) *Mode of Distribution*

The Conference recommends that the International Standard Preparation shall be issued in the form of a standard solution in oil, the strength of the solution being such that 1 gramme contains 500 International units, or 300 micrograms (300γ) of β-carotene. (See Note 2.)

(e) *Adoption of a Subsidiary Standard of Reference*

The Conference recommends that a sample of cod liver oil, the potency of which has been accurately determined in terms of the International Standard Preparation of β-carotene, shall be provided as a Subsidiary Standard of Reference.

In view of the fact that the Reference Cod Liver Oil of the

United States Pharmacopœia, which has been accurately assayed in terms of the provisional International Standard adopted in 1931, has been in effective use in the United States of America for some time, the Conference recommends that the Board of Trustees of the United States Pharmacopœia be approached and invited to place a quantity of their Reference Cod Liver Oil at the disposal of the Health Organisation of the League of Nations, with a view to its adoption for International use as a subsidiary standard for vitamin A.

In the event of the Reference Cod Liver Oil of the United States Pharmacopœia not being available for International adoption, the Conference recommends that another sample of cod liver oil be selected, its potency in terms of the International Standard Preparation of β-carotene accurately determined by biological comparison and independently by spectrophotometric measurements (see (f) below), and that this selected sample be then adopted as a Subsidiary International Standard for vitamin A.

(f) *Spectrophotometric Test for the Assay of Vitamin A in Liver Oils and Concentrates of High Potency*

It has been found that, within certain defined conditions, measurements of the coefficient of absorption (E) at 328 mμ affords a reliable method for measuring the vitamin A content of liver oils and concentrates. As a means of converting values obtained for $E^{1\%}_{1cm.}$ 328 mμ into a figure representing the International units of vitamin A per gram of the material examined, the factor 1600 is recommended for adoption. (See Note 3.)

Note 1

Properties of Pure β-Carotene ($C_{40}H_{56}$).

(*a*) Melting-point, 184–184·5° C., corrected.
(*b*) Optically inactive.
(*c*) Absorption spectrum showing bands—
in carbon disulphide at 519, 485·5 mμ
in chloroform at 495, 465 mμ
in cyclohexane at 486, 456 mμ.

Note 2

Nature of the Oil to be used as Diluent.

The oil used for the preparation of the Standard solution of β-carotene, and for the further dilution of the Standard and for use in the biological assay of vitamin A, shall be a colourless, vitamin A-free, vegetable oil, from which the dissolved carotene can be properly absorbed from the alimentary canal of the test animals. 0·01% of hydroquinone shall be added to the oil to prevent oxidation and ensure stability.

In order to establish its suitability, the oil to be used as diluent for the above-named purposes must be submitted to the following test: A 0·003% solution of β-carotene in the oil is prepared, and the colour of the solution compared with that of a 0·5% solution of potassium bichromate; about 6 cc.

of the carotene solution in oil are placed in a flat-bottomed tube (5 cm. × 2 cm.), which is closed with a hard cork so as to leave an air space of about 6 cc. capacity. The tube is then maintained at a temperature of 37° C. in the dark for 7 days, and the colour of the solution again compared with that of the Standard solution of potassium bichromate. An oil may be considered satisfactory for the purposes indicated if the loss in colour does not exceed 10%. Some samples of cocoa-nut oil have been found to fulfil the requirements of the above test.

Note 3

The Spectrophotometric Test.

(*a*) The measurement must be made on the unsaponifiable fraction, unless the material is of potency greater than 10,000 International units per gram.

Method of saponification.—Since the error associated with the saponification may be considerable, the following method is recommended, as yielding trustworthy results.

One gram of oil is saponified with 10 cc. N/2 freshly prepared alcoholic KOH, by boiling until clear (time needed about 5 minutes). 20 cc. water are added, the whole transferred to a small separator and extracted with two quantities of 25 cc. ether (peroxide-free). The ethereal extracts are washed first with water (10–20 cc.), then with 10–20 cc. N/2 KOH, and again with water, while rotating gently without shaking. The ethereal solution is then shaken thoroughly with two quantities of 10 cc. water, after which it is filtered into a flask, the ether evaporated almost to dryness and the residue dissolved in ethyl alcohol or cyclohexane and made up to the concentration required for the particular instrument in use. A preliminary test on the original oil will indicate the amount both of oil and of solvent which will be necessary.

(*b*) Pure ethyl alcohol or cyclohexane must be the solvent used. Pure cyclohexane, suitable for spectrographic examination, should have the following properties: $d^{20°}_{4°}=0{\cdot}7784$; B.P. 81·4° C.; F.P. 6·5° C.; it should be almost completely transparent in the region of 328 mμ and exhibit no trace of discontinuous absorption.

(*c*) The intensity of absorption at 328 mμ may be determined to within ±2·5% by any of the recognised methods of spectrophotometry.

(*d*) The factor, 1600, is the average figure derived from a series of comparative and independent tests on the unsaponifiable fractions of liver oils and on concentrates of high potency. It is desirable that, when a figure expressing the biological potency of a preparation has been derived by the use of this calculation, the fact should be stated.

II. Vitamin B_1

(a) *International Standard*

The Conference confirms the adoption, as International Standard, of the adsorption product of vitamin B_1 prepared in the Medical Laboratory, Batavia (Java), by the method of Seidell, as described by Jansen and Donath.

(b) *Terminology*

The International Standard Preparation shall be known as the "standard adsorption product of vitamin B_1."

(c) *Method of Preparation*

The International Standard was prepared by extracting rice polishings with water, sufficient sulphuric acid being added to make

the *p*H 4·5. Salicylic acid to a concentration of 0·2 per cent. and toluene were then added to prevent bacterial decomposition. The process of extraction was continued for two days, after which the solution was filtered. For each 100 kg. of the original rice polishings 3 kg. of fuller's earth (specially selected for its adsorptive powers) were added to the solution, which was then stirred for 24 hours. Subsequently, the solution was filtered off and the fuller's earth, after being washed with water and alcohol, was dried ; 3 kg. of the fuller's earth adsorbate represents the vitamin B_1 from 100 kg. of rice polishings.

(d) *Definition of Unit*

The International unit recommended for adoption is the vitamin B_1 activity of 10 milligrammes of the International Standard adsorption product.

A daily dose of 10–20 milligrammes of this preparation is required to maintain normal growth in a young rat on a diet deficient in vitamin B_1 but complete in all other respects. The curative " day dose " for a pigeon (300g. weight) exhibiting head retraction on a diet of polished rice is about 10–30 milligrammes (method of Kinnersley and Peters). The Standard contains vitamin B_4, but relatively small amounts of vitamin B_2.

(e) *Mode of Distribution*

The International Standard adsorption product of vitamin B_1 is kept at the National Institute for Medical Research, London, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations.*

No special precautions are necessary in keeping this preparation, except that it should be stored in a dry place. In the presence of moisture, bacterial decomposition readily takes place.

(f) *Recommendations for Future Research*

The potency of the Standard adsorption product should be tested relatively to that of the crystalline vitamin B_1 preparations at present available, with the aim of ultimately adopting pure crystalline vitamin B_1 as International Standard. The following, who have isolated such crystalline preparations, should be asked to make this comparison, employing the biological methods usual in their own laboratories :

Professor B. C. P. JANSEN, Amsterdam.
Professor R. A. PETERS, Oxford.
Dr. A. SEIDELL, Washington.
Professor U. SUSUKI, Tokio.
Dr. R. R. WILLIAMS, New York.
Professor A. WINDAUS, Göttingen.

In further research, the comparative effect of parenteral and oral administration should be examined. In the case of the Standard adsorption product, the preparation of suitable extracts should be investigated.

III. VITAMIN C

(a) *International Standard*

The Conference recommends the adoption of l-ascorbic acid as International Standard. The Standard Preparation shall conform to the requirements stated in Note 4, below, in regard to its chemical and physical constants.

(b) *Definition of Unit*

The unit recommended for adoption is the vitamin C activity of 0·05 milligramme *l*-ascorbic acid.

This is about one-tenth of the daily dose necessary to prevent development of gross macroscopic scorbutic lesions in a young guinea-pig maintained on a scurvy-producing diet.

(c) *Mode of Preparation*

It is recommended that the Institute of Medical Chemistry, Szeged, should be asked, through Professor SZENT-GYÖRGYI, to prepare a batch of 500g. of the Standard Preparation, and that Professor W. W. HAWORTH, University of Birmingham, be invited to co-operate in controlling the purity of the Standard material.

(d) *Place of Distribution*

It is recommended that the National Institute of Medical Research, London, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations*, shall be requested to undertake the storage and distribution of the vitamin C standard.

(e) *Method of Use*

The Standard may be kept at room temperature in a sealed tube or in an evacuated desiccator, and the required amount must be dissolved in freshly boiled and cooled glass-distilled water, immediately before daily administration to the test animals.

Note 4

Properties of the Vitamin C Standard.

The Standard Preparation of *l*-ascorbic acid should have the following characteristics:

(*a*) Melting-point 192° C., uncorrected, taken in an open capillary, without appreciable darkening before melting.

(*b*) Specific rotation $[a]_D^{20°} = +20·0°$ in water (conc. 14·0g. per 100 cc. solution)

$[a]_D^{20°} = +22·4°$ in water (conc. 2·2g. per 100 cc.

(*c*) The absorption spectrum in slightly acid aqueous solution is characterised by a single intense band with head at 245 mμ. The molecular extinction coefficient for a solution containing 20 milligrammes per litre is 10,000. (The ascorbic acid is to be dissolved in freshly distilled water to which 0·1 cc. N/1 sulphuric acid has been added for every 100 cc. water).

(*d*) 10 milligrammes *l*-ascorbic acid should require 11·4 cc. N/100 aqueous iodine, the titration being carried out with starch as indicator.

(*e*) The substance must be ash-free and its elementary analysis must agree with the figures for $C_6H_8O_6$. It normally crystallises in rectangular plates which show straight extinction with high birefringence. [nα (D line) 1·47±0·005 ; nβ (D line) 1·68±0·005].

IV. Vitamin D

(a) *International Standard*

The Conference recommends that the Standard solution of irradiated ergosterol, prepared at the National Institute for Medical Research, London, and at present issued as International Vitamin D Standard, shall be retained.

When the present International Standard solution is exhausted, or if it should become unsatisfactory for any reason, the Conference recommends that it should be replaced by an equivalent solution of pure crystalline vitamin D in olive oil, of such strength that 1 milligramme contains 0·025 micrograms (0·025γ) of crystalline vitamin D. (For a definition of crystalline vitamin D, see Note 5 below.)

(b) *Definition of Unit*

The unit of vitamin D recommended for adoption by the 1931 Conference shall remain unaltered : namely, the vitamin D activity of 1 milligramme of the International Standard solution of irradiated ergosterol, which has been found equal to that of 0·025 micrograms (0·025γ) of crystalline vitamin D.

The assay of materials for vitamin D content should be carried out by comparative tests on rats, and their value in International units should be derived from the results of such tests. If other species are employed for these tests, the values cannot be expressed in International units (see (d) below).

(c) *Mode of Distribution*

The National Institute for Medical Research, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations*, shall be asked to continue the storage and distribution of the International Vitamin D Standard. The mode of distribution recommended by the 1931 Conference shall remain unaltered either in the case of the existing Standard solution, or the solution of crystalline vitamin D by which it may be replaced in future.

(d) *Recommendations for Further Research—Cod Liver Oil as Subsidiary Vitamin D Standard*

The Conference recognises that amounts of cod liver oil and irradiated ergosterol which contain an equal number of vitamin D units, as determined by tests with rats, may not possess equal anti-rachitic activity when tested on certain other species—*e.g.* poultry. The utility of cod liver oil as a secondary standard of reference is obvious, and such standard cod liver oils are at present distributed in several countries. In view of the fact that inconveniently large quantities of cod liver oil would have to be handled for International purposes as Vitamin D Standard, thereby giving rise to storage problems, the Conference does not recommend the adoption of a sample of cod liver oil as a subsidiary International Standard for Vitamin D.

The Conference recommends, however, that national centres of distribution should prepare subsidiary reference samples of cod liver oil, of known vitamin D activity, for distribution to workers in the countries concerned. In order to preserve a uniform value for the International unit, the vitamin D activity of such oils should be compared with that of the International Standard by means of tests on rats and their potency should be stated in International units. It is recommended that the experience gained with such subsidiary cod liver oil standards should be devoted to an attempt to elucidate the questions involved in the anomalous action on certain species of different sources of vitamin D, and that members of the Conference be asked to co-operate in these investigations in their respective countries.

(e) *Biological Methods for Estimation of Vitamin D*

In using the International Standard Solution for the determination of the anti-rachitic potency of unknown preparations, it is recommended that not fewer than 20 similar rats (preferably more) be used for a determination, half of these to receive the Standard and the remaining litter-mates the unknown substance. Provided this precaution is observed, it is considered permissible to use either prophylactic or therapeutic methods of investigation.

Note 5

Properties of Crystalline Vitamin D (Calciferol or Vitamin D_2), $C_{28}H_{43}OH$.

(*a*) Colourless acicular crystals, odourless; melting-point, 114·5–117° C. (open capillary).

(*b*) Specific rotation:

in alcohol $[\alpha]_D^{20^\circ} = +101^\circ$ to $+102{\cdot}5^\circ$; $[\alpha]_{5461}^{20} = +119^\circ$ to $+122^\circ$

in chloroform $[\alpha]_D^{20^\circ} = +52^\circ$; $[\alpha]_{5461}^{20} = +62^\circ$.

(*c*) Absorption spectrum: in alcohol or other suitable non-absorbing solvent, a smooth curve with a maximum at 265 mμ $E_{1cm.}^{1\%} = 470$–485).

APPENDIX II

VITAMIN CONTENT OF SOME FOODS EXPRESSED AS INTERNATIONAL UNITS PER GRAM

Determined by direct comparison with the International Standard in the Laboratory of the Pharmaceutical Society

Vitamin A

Halibut liver oil, 30,000–360,000—		Egg yolk	30
5 samples, average ..	160,000	Bone marrow	8
Cod liver oil, 600–4,000—		Carrots, fresh or boiled ..	19
26 samples, average ..	1,950	Cabbage, fresh or boiled ..	9
Pasteurised milk—		Runner beans, fresh or boiled	6
(*a*) November, 1934 ..	3	Tomato, fresh (no skin or seeds)	30
(*b*) May, 1936 ..	5·5	Apple, Bramley Seedlings ..	0·4
(*c*) October, 1936 ..	3·5	Orange juice	3
(*d*) February, 1937 ..	0·7	Vegetable marrow	0·3
Jersey milk—		Wheat germ	6·5
(*a*) November, 1934 ..	5	Peas, uncooked	7
(*b*) May, 1936 ..	7	Beetroot, cooked	0·5
(*c*) October, 1936 ..	4·5	Spinach, uncooked	130
(*d*) February, 1937 ..	1·4	Celery, uncooked	0·5
Butter, 8–200—		Parsnip, uncooked	2
36 samples, average ..	53	Banana	0·8

Vitamin B_1

Dried yeast (*a*)	34	Wheat embryo	11
(*b*)	24	A Swiss " full corn " bread	1·4
(*c*)	38		
(*d*)	120		
(*e*)	20		
(*f*)	7		

Vitamin D.

Halibut liver oil, 2,000–4,000—		Cream	0·5
10 samples, average ..	2,450	Egg yolk (*a*)	1·5
Cod liver oil, 60–300—		(*b*)	5·0
439 samples, average	126	Calf liver, fresh (June) ..	nil
Milk, 20 samples, 0–0·1		even when tested as 10% of diet.	
Butter, 0·0–4·0—		Olive oil	nil
26 samples, average ..	0·9	even when tested as 20% of diet.	